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The Influence of Hybridization Upon the Natural Resistance of Mice to the Progressive Growth of Sarcoma 37

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Successful growth of a propagable neoplasm is largely dependent upon the constitution of the inoculated animal. Tyzzer (*1*) and Loeb (*2*) drew attention to the importance of this factor when they found that tumors arising in one stock of mice grew in other members of the same stock but not in members of an unrelated stock. Tyzzer (*3*) extended the work by showing that a tumor occurring in one stock grew in first-generation hybrids derived from that stock.

The development of inbred strains of animals made possible a more thorough study of the relationship between the host and implanted tumor tissue. Little, Strong, Bittner, Cloudman, and others found that successful growth of transplanted neoplastic tissue depended upon the genetic relationship of the host and the tumor. A tumor occurring in an individual of an inbred strain of mice grew in all other members of the same strain but not in members of another inbred strain. Furthermore, the number of Mendelian factors involved in the progressive growth of a transplanted tumor was determined by the use of appropriate hybrid animals. Bittner (*4*) has reviewed the contributions to this subject made by geneticists.

In this laboratory (*5*) certain inbred strains of mice have been tested for their susceptibility to spontaneous, induced, and transplantable tumors. In an earlier paper (*6*) dealing with the natural resistance of these mice to the growth of two transplantable neoplasms, it was shown that the cutaneous growth of the tumors was a better test for the natural resistance of the inoculated animals than the subcutaneous growth, since the results were more clear-cut with the former method.

The cutaneous-inoculation technique revealed that in practically all mice of strains L,[1] C57 Black, C57 Brown, I, and C3H, sarcoma 37 regressed spontaneously, and in practically all members of strains Y, C, and D it grew progressively and killed its

[1] *This strain was designated as strain M in the earlier paper* (6), *but the designation has been changed since that time.*

hosts. In strain A the tumor grew progressively in about 20 percent of the animals and regressed in the remainder. When sarcoma 180 was implanted cutaneously in the same strains, only the mice of strain I possessed sufficient natural resistance to make the tumor regress.

Further studies on the natural resistance of these strains of mice and their hybrids are reported here. It was especially desirable to ascertain the susceptibility of hybrids since most of the previous work (*4*) was performed with tumors that arose within inbred strains or hybrids closely related genetically to the test animals. In the present studies the tumor employed was a well-known propagable growth which was described before the inbred lines of mice were established.

MATERIALS AND METHODS

Mice of strains L, C57 Black, Y, C, I, A, and D were used. A description of these strains and their degree of inbreeding was presented in the earlier paper (*6*). All were inbred for at least 20 generations and some for many more. Animals of strains L, C57 Black, A, and D were obtained from the Roscoe B. Jackson Memorial Laboratory, while those of strains Y, C, and I were raised in this laboratory.

Most of the animals were inoculated when 2 to 3 months of age, but those used as breeders were 4 to 5 months old. All mice were fed the same diet (Purina dog chow) and kept under similar environmental conditions.

The well-known inoculable mouse sarcoma 37 was used. This tumor was first described by Haaland (*7*) in 1908 as a rapidly growing tumor which could be propagated in ordinary "market" mice. It has been propagated in this laboratory during the past 10 years and during the past 7 years has been grown exclusively in the subcutaneous tissues of strain D mice. Tumors growing in this strain were used to inoculate all the animals used in these investigations.

Most tumor implantations were made immediately under, and as close as possible to, the epidermal layer of the skin and are referred to as cutaneous inoculations. The criteria for successful inoculation as well as the details of technique were described previously (*6*). The technique consists of passing viable 10-day-old tumor tissue through a fine mincer so that the minced tissue will pass through an 18- or 20-gage needle. A 1-cc. tuberculin syringe is used in which the slip opening is enlarged to permit passage of the inoculum. Implantation is made into the shaved skin over the abdomen by inserting the needle just beneath the outer layer of skin so that its bevel is plainly visible and expelling a small amount of the minced tissue. One cubic centimeter of inoculum is sufficient for 20 to 25 inoculations.

EXPERIMENTAL OBSERVATIONS

Results in Inbred Strains

The results of all cutaneous inoculations of inbred strains performed since the publication of the first paper are summarized in table 1. The last implantations were made during February 1941. Mice of strains Y and D developed rapidly growing tumors which grew progressively and killed their hosts. In C57 Black and L mice the tumor grew rapidly for 12 to 14 days and then regressed completely in virtually all the animals. The transplanted tumor grew much more slowly in mice of strains C and I than in the other strains. In strain I it grew for 12 to 14 days and then regressed completely. In strain C, however, the tumor continued to grow slowly and ultimately killed the

TABLE 1.—*The results of cutaneous inoculation of inbred strains of mice with sarcoma 37*

Strain	Sex	Mice inoculated		
		Number	Susceptible	Resistant
			Percent	*Percent*
L	F	41	0	100
L	M	42	7.1	92.9
C57 Black	F	37	0	100
Do	M	54	1.8	98.2
Y	F	23	100	0
Y	M	36	100	0
C	F	42	100	0
C	M	46	100	0
I	F	37	0	100
I	M	44	0	100
A	F	39	77	23
A	M	70	70	30
D	F	28	100	0
D	M	27	100	0

animals. It is clear that the rate of growth of sarcoma 37 during the first 2 weeks is no indication of its ability to continue progressive growth and to kill the host.

Strain A mice were given special consideration since they were the only animals which did not react uniformly in the previous studies. An effort was made to ascertain whether the strain responded uniformly to the inoculation of another type of neoplasm. This tumor was a spontaneous mammary-gland adenocarcinoma arising in a strain A female.[2] It was inoculated into the subcutaneous tissues of 61 (18 female and 43 male) strain A mice by the usual trocar technique and grew progressively in every animal. Cutaneously inoculated sarcoma 37, however, grew progressively in 30, or 79 percent of 38 male litter mates of the same mice and regressed in the remaining 21 percent. This indicated that strain A mice were a homozygous strain so far as susceptibility to 1 spontaneous mammary tumor was concerned, but when inoculated cutaneously with sarcoma 37 they exhibited pronounced differences in their degree of natural resistance.

[2] *Kindly supplied by Dr. J. J. Bittner.*

RESULTS IN HYBRID MICE

Two series of hybrid mice were derived from three of the inbred strains. Resistant strain I and susceptible strain Y mice were used to obtain the first series. Representatives of the strains were mated reciprocally to procure the F_1 generation of hybrids, which were then mated brother to sister to obtain the F_2 generation or were mated reciprocally to the parent stocks to obtain the backcross generations. Every mouse was inoculated cutaneously with sarcoma 37, which regressed in every strain I animal and grew progressively in every strain Y mouse. The results in the hybrid generations are shown in table 2. The tumor grew progressively in all F_1 generation and Y-backcross-generation mice, in 82 percent of the F_2 generation, and in approximately 50 percent of the I-backcross generation.

The second series of hybrids were derived from resistant strains I and L. As in the first series reciprocal matings were made to produce the F_1 generation. The mice of this generation were mated brother to sister to give the F_2 generation and were mated reciprocally to the parent strains to obtain the backcross generations. Every mouse was inoculated cutaneously with sarcoma 37, which regressed

TABLE 2.—*Results of cutaneous inoculation of hybrid mice derived from strains Y [1] and I [2], with sarcoma 37*

Generation	Sex	Mice inoculated		
		Number	Susceptible	Resistant
			Percent	*Percent*
F_1	F	51	100	0
F_1	M	39	100	0
F_2	F	48	84	16
F_2	M	49	80	20
I backcross	F	153	48.4	51.6
Do	M	131	48.9	51.1
Y backcross	F	45	100	0
Do	M	44	100	0

[1] 100-percent susceptible.
[2] 100-percent resistant.

completely in all strain I and strain L mice. The results in the hybrid mice are summarized in table 3. The tumor grew progressively in about 87 percent of the F_1 generation, in about 60 percent of the F_2 generation, and in approximately 80 and 30 percent of the L-backcross and I-backcross mice, respectively.

DISCUSSION

The results in the inbred strains reveal that they continue to exhibit the same degree of natural resistance to progressive growth of cutaneously inoculated sarcoma 37 as they did 4 years ago. Strains I, C57 Black, and L are resistant, while strains Y, C, and D are susceptible. Strain A may be designated as an intermediate strain. It is significant that such clear-cut results are obtainable with sarcoma 37 which did not arise spontaneously in any of the strains. It will be recalled that this tumor originated in 1908, and before inbreeding had established any of the strains.

The variation in susceptibility to cutaneous growth of sarcoma 37 as exhibited by different strains, together with the temporary growth followed by complete regression in certain strains, offers an opportunity for chemical or histologic studies in the problem of growth and regression of transplantable tumors.

In the previous paper (*6*) it was shown that the animals in which cutaneous growths had receded spontaneously were immune to subsequent subcutaneous inoculations of the same tumor. The cutaneous-inoculation technique is therefore an excellent method for procuring actively immunized mice.

The primary object of the present investigation was to ascertain the susceptibility of hybrid mice derived from inbred strains, and the outcome of these studies was of some interest. Hybrid mice obtained from resistant strain I and susceptible strain Y produced results which suggested that progressive growth of the tumor was controlled by a single dominant factor. This interpretation is supported by the 100-percent susceptibility of the F_1 and Y-backcross generations and by the 50-percent susceptibility of the I-backcross generation. Further work is essential, however, before a definite conclusion can be made.

The results of hybridization studies between resistant strains I and L were unexpected. If susceptibility is inherited as a single dominant factor, as suggested by the I by Y hybrids, it might be expected that F_1 hybrids derived from two resistant strains would be resistant. But, as shown in table 3, approximately 85 percent of the F_1 mice were susceptible. This outcome cannot be attributed to any change in the growth characteristics of sarcoma 37, for throughout the study hybrids from strains I and Y and those from strains I and L were inoculated with the same tumor material.

An increase in susceptibility due to hybridization is not limited to transplantable tumors only, since previous work in this laboratory (*8, 9*) showed that

TABLE 3.—*Results of cutaneous inoculation of hybrid mice derived from strains* [1] *I. and I, with sarcoma 37*

Generation	Sex	Mice inoculated		
		Number	Susceptible	Resistant
			Percent	*Percent*
F_1	F	37	86.5	13.5
F_1	M	40	87.5	12.5
F_2	F	60	53.3	46.7
F_2	M	61	62.3	37.7
L backcross	F	39	71.8	28.2
Do	M	51	88.2	11.8
I backcross	F	32	28.1	71.9
Do	M	36	33.3	66.7

[1] Both strains 100-percent resistant.

hybridization of strains C3H and I resulted in offspring which were more susceptible to induced pulmonary tumors than either parent strain. It was also noted that hybrids derived from strains C3H and Y were more resistant to the development of spontaneous mammary-gland tumors than the susceptible C3H strain. Such findings, together with those presented here, permit the conclusion that although the susceptibility of the parent stocks is well established, the susceptibility of hybrid mice to tumor growth cannot be predicted.

In these transplantation experiments the results obtained from strains I and Y and their hybrids indicate that these two strains may differ by a single pair of factors controlling progressive growth of the tumor, the factor for susceptibility being dominant over that for resistance. However, before such a single-factor hypothesis can be definitely proved, breeding tests of the F_2 or backcross segregants are essential, especially in view of the results obtained from strains I and L and their hybrids. These results indicate that strains I and L differ by more than a single pair of factors for susceptibility. The degree of susceptibility of the F_1 hybrids which was higher than that of either parent strain was due to a summation of susceptible factors. This indicates the inadvisability of using but one series of hybrids and formulating a general conclusion regarding the inheritance of susceptibility to tumor growth. Such concluclusions must be limited to the particular series of animals and their parent stocks.

SUMMARY AND CONCLUSIONS

Mice of inbred strains L, C57 Black, Y, C, I, A, and D were tested for natural resistance to progressive growth of cutaneously inoculated sarcoma 37. The tumor grew progressively and killed all mice of strains Y, C, and D but regressed completely in virtually all mice of strains L, C57 Black, and I. It grew progressively in 75 percent of strain A mice and regressed in the remainder. These results were in harmony with those obtained in 1937 and indicate that neither the susceptibility of the mice nor the growth characteristics of the tumor had changed during the last 4 years.

In hybrid mice derived from resistant strain I and susceptible strain Y the tumor grew progressively in all F_1 generation and Y-backcross-generation animals, in 82 percent of the F_2 generation, and in approximately 50 percent of the I-backcross generation.

In hybrid mice derived from resistant strains I and L the tumor grew progressively in about 87 percent of the F_1 generation, in about 60 percent of the F_2 generation, and in approximately 80 and 30 percent of the L-backcross and I-backcross mice, respectively.

These results in the hybrid generation permit the conclusions that (1) the natural resistance of the parent strains gives no indication of the degree of natural resistance of their hybrids, and (2) one series of hybrids should not be used for formulating any general conclusions regarding inheritance of natural resistance.

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The Influence of the Paternal Parent in Determining the Susceptibility of Mice to Spontaneous Mammary Tumors

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The occurrence of spontaneous mammary cancer in mice is controlled by at least two factors: (1) The presence of an extrachromosomal (nongenetic) influence which is transmitted in the milk of the mother; and (2) the inherited (genetic) susceptibility of the mice to the action of the milk influence. Murray (*1*, *2*) gave this problem extensive consideration and concluded that while the extrachromosomal influence was 10 times as powerful as any chromosomal factors in first-generation hybrids, it became noneffective in later generations. Starting with first-generation hybrids derived by mating females of a susceptible strain to males of a resistant strain he procured 8 generations of backcross animals by mating females of each successive generation to resistant-strain males. This procedure permitted the transmission of the milk influence to each generation and concentrated the chromosomal influence of the resistant strain until the final backcross generation had practically the genetic constitution of the resistant strain. He found that the incidence of mammary cancer decreased rapidly in the successive backcross generations. Van Gulik and Korteweg (*3*) performed the same experiment to the eleventh backcross generation and obtained similar results. Such observations emphasized the importance of the genetic constitution of the backcross mice in determining one of the following possibilities: the susceptibility of their mammary tissues to the action of the milk influence, their ability to inactivate the milk influence, or their inability to propagate the milk influence. A test for the presence of the milk influence in the backcross generations was not reported from either laboratory but Van Gulik and Korteweg stated that, "The extrachromosomal factor becomes inactive after a number of generations when the chromosomal factor is not present at the same time."

The same investigators also procured the reciprocal series of backcross animals by mating females of a resistant strain to males of a susceptible strain and mating females of each successive generation to susceptible-strain males. This procedure eliminated the milk influence and concentrated the chromosomal factors of the susceptible strain until the final backcross animals had practically the genetic constitution of the susceptible strain. Very few mammary tumors arose in the backcross generations which demonstrated that the presence of chromosomal factors alone was not sufficient for the occurrence of mammary tumors.

Bittner also studied the genetic factors involved in susceptibility to mammary cancer in mice. He mated susceptible-strain females to resistant-strain males and obtained first-generation hybrids which were mated brother to sister to procure the F_2 or second-generation hybrids.

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Bittner (*4*) summarized his studies on foster nursing in a recent communication and concluded that, "Breast tumor ratios observed in hybrid animals were in accord with the genetic theory that breast-cancer susceptibility is transmitted as a single dominant factor." In a later paper (*5*), he referred to the average age of occurrence of mammary cancer in mice and stated that, "The average tumor age may be independent of the inherited susceptibility factor or factors and be governed by modifying factors." It is assumed that Bittner has not come to any definite conclusion concerning the number of genetic factors involved.

Previous work in this laboratory (*6*, *7*) also showed that chromosomal factors play an important role in the genesis of mammary tumors in mice. First-generation hybrids derived by breeding strain C3H females to strain I males were far more susceptible to the development of mammary tumors than those obtained from strain C3H females and strain Y males. This established the importance of a paternal influence [1] in controlling the degree of susceptibility of the hybrids.

The purpose of this paper is to report two experiments in which the breeding was carried beyond the first hybrid generations to observe whether the paternal parent exerted any influence in later generations. The first investigation was similar to those of Murray and of Van Gulik and Korteweg in that backcross mice were used, and the second paralleled Bittner's studies by obtaining second-generation hybrids.

EXPERIMENTAL PROCEDURE

Mice of strains C3H, I, Y, and C57 Black were used. All were raised in this laboratory and were descendants of litters procured from the Roscoe B. Jackson Laboratory. Females of strain C3H are highly susceptible (*8*) to the development of mammary cancer, while those of strains I, Y, and C57 Black are very resistant (*9*). All experimental animals were kept under similar environmental conditions and on a diet of Purina dog-chow pellets with an unlimited supply of water.

The first experiment was begun during November 1938. Strain C3H females were mated to strain I males, and females procured from this mating were bred to strain I males to obtain the first I-backcross generation. Females of the first I-backcross generation were then mated to I males to obtain the second I-backcross generation. First and second Y-backcross generations were derived by using Y

[1] *The term "paternal influence" throughout this paper does not imply chromosomal influences transmitted only through the male parent. The female parent may also transmit these influences to her offspring.*

TABLE 1.—*The occurrence of spontaneous mammary tumors in mice of the F_1 generation and two backcross generation obtained from parental strains C3H, I, and Y*

Parents	Hybrid generation	Group	Number	Tumor incidence	Average tumor age	Average age at death of mice dying without tumor
				Percent	*Months*	*Months*
C3H ♀ × I ♂	F_1	Virgins	5	100	10.3	
C3H ♀ × I ♂	F_1	Breeders	7	100	7.7	
C3H I F_1 ♀ × I ♂	First I backcross	do	13	69.2	10.7	19.4
First I backcross ♀ × I ♂	Second I backcross	do	31	12.9	10	15.4
C3H ♀ × Y ♂	F_1	Virgins	5	80	15	11
C3H ♀ × Y ♂	F_1	Breeders	7	71	14.4	23.2
C3H Y F_1 ♀ × Y ♂	First Y backcross	do	19	15.8	14.5	18
First Y backcross ♀ × Y ♂	Second Y backcross	do	54	7.4	12.6	15.3

males as breeders. All mice of the first- and second-backcross generations bore one litter. Mice of the first generation raised their litters, but litters of the second-generation mice were killed within 24 hours after birth. Few animals were obtained in the first generations because interest in the investigation was centered in later backcrosses.

The results are summarized in table 1. The F_1 generation hybrids derived from C3H females and I males were more susceptible to the development of mammary cancer than those obtained from C3H females and Y males. The first I-backcross generation had a tumor incidence of 69.2 percent at an average age of 10.7 months, while the first Y-backcross generation showed an incidence of 15.8 percent at an average age of 14.5 months. Mice of the I-backcross generation were therefore more susceptible than those of the Y-backcross generation.

Both second-backcross generations gave a low incidence of mammary tumors which indicated that a continuation of the experiment probably would have given results similar to those of Murray and of Van Gulik and Korteweg, that is, continued backcrossing to the resistant strains would have produced increasingly resistant mice.

The second experiment was begun during March 1939. Strain C3H females were mated to males of strain I or C57 Black, and the first hybrid generations (F_1) thus obtained were mated brother to sister to procure the F_2 generations. All F_1 mice raised one litter, while the F_2 hybrids bore three litters in rapid succession; each litter was killed within 24 hours after birth.

A summary of the results is presented in table 2. The F_1 hybrids derived from C3H females and I males developed tumors at an average age of 8.4 months,

TABLE 2.—*The occurrence of spontaneous mammary tumors in mice of the F_1 and F_2 generations obtained from parental strains C3H, I, and C57 Black*

Parents	Hybrid generation	Number	Tumor incidence	Average tumor age	Average age at death of mice dying without tumor
			Percent	*Months*	*Months*
C3H ♀×I♂	F_1	26	100	8.4	
C3H I F_1 ♀×C3H I F_1♂	F_2	43	86	8.8	13.6
C3H ♀×B[1]♂	F_1	21	95.5	11.9	23
C3H B[1] F_1 ♀×C3H B[1] F_1♂	F_2	43	74.4	11.2	15

[1] B denotes strain C57 Black mice.

and those obtained from C3H females and C57 black males did so at an average age of 11.9 months. This established the higher susceptibility of the C3H×I outcross.

F_2 hybrids obtained from strains C3H and I gave a tumor incidence of 86 percent at an average age of 8.8 months while those derived from strains C3H and C57 Black had a tumor incidence of 74.4 percent at an average age of 11.2 months. This indicated the greater susceptibility of the C3H×I hybrids.

A group of the F_2 hybrids, not included in the table, were used in another experiment. These were virgin mice which at the age of 6 weeks were inoculated subcutaneously in the right axilla with a 3-mg. pellet of fused crystals of estrone. The dose of estrogen was too large and killed most of the animals; none survived more than 4 months after the injection. In that time, however, of 35 F_2 hybrids derived from strains C3H and I, 9, or 25.7 percent, developed mammary cancer, while of 28 F_2 hybrids obtained from C3H and C57 Black, only 1, or 3.6 percent, developed a similar growth. When mice dying before the appearance of the first tumor are excluded, the C3H×I hybrids gave an incidence of 42.9 percent and the

C3H×C57 Black an incidence of 7.7 percent. This result again indicated the higher degree of susceptibility of the C3H ×I hybrids.

DISCUSSION

These investigations reveal that genetic factors are of considerable importance in the occurrence of mammary cancer in mice in that they determine the susceptibility of the mouse to the nongenetic influences which are present in the milk of the mother.

In both experiments tumors arose in first-generation hybrids obtained by mating females of a high-tumor strain to males of resistant strains, and it was shown that genetic factors derived from resistant-strain fathers were important in determining the degree of susceptibility of the hybrids. In this respect the outcome of the experiments confirmed previous observations made in this laboratory.

In the present studies, however, additional hybrid generations were obtained in order to ascertain whether the paternal influence could also be demonstrated. In the first experiment, first-backcross generations were obtained by mating females of the first hybrid generations to resistant-strain males and it was found that the paternal influence was effective in the backcross mice; those obtained by backcrossing to strain I males were more susceptible than those derived by backcrossing to strain Y males. In a further backcross generation the difference in susceptibility was less pronounced because of the low incidence of tumors in both groups. This low incidence in the second-backcross generations was due to an increase of resistant-strain chromatin which may have been manifested in the inactivation of the milk influence, or the inability of the mice to produce the influence, or the increased resistance of the mammary tissues.

The incidence of tumors in the various groups of hybrid mice of both experiments permits the conclusion that in these mice susceptibility to the development of mammary cancer was influenced by chromosomal factors which were introduced from the paternal side. The fact that the chromosomal influence from the I males differed from that from the Y or C57 Black males indicates that multiple genetic factors were involved. To prove that a single factor was involved would require the use of one strain which was completely resistant to this type of tumor, and none of the strains fulfilled this requirement. Indeed, it is doubtful if a strain of mice has thus far been established which is completely free from the development of mammary-gland tumors. Murray (*2*) has discussed the value of inbred strains of mice in cancer research and stated that, "The study of these stocks has shown them to be subject to the development of spontaneous mammary-gland carcinomas in greater or lesser degree." Furthermore, it is known (*10*) that mammary tumors arise in resistant-strain mice when foster nursed by females from susceptible strains; when fostered by females of strain C3H from 15 to 20 percent of C57 Black females and over 65 percent of strain C females develop mammary tumors. This implies a difference in degree of susceptibility to the milk influence.

There are a number of inbred strains of mice of different genetic constitutions; but since all are susceptible to the development of mammary cancer, it is probable that a number of factors, both genetic and nongenetic, are involved in the genesis of this type of tumor and that the genetic factors exert their influence by controlling the degree of susceptibility.

SUMMARY

Female mice of high-tumor strain C3H were mated to males of low-tumor strains I or Y to procure mice of the F_1 generation. Females of the F_1 generations were mated to strain I or Y males to obtain first-generation-backcross mice and females of this backcross were mated to I and Y males to obtain second-generation-backcross mice.

In both the F_1 and first-backcross generations mice derived from strains C3H and I were more susceptible to spontaneous mammary tumors than those derived from strains C3H and Y. The incidence of mammary tumors was low in both second-backcross generations.

Female mice of strain C3H were mated to males of low-tumor strains I or C57 Black, and the F_1 hybrids thus obtained were mated brother to sister to procure the F_2 generations. Both the F_1 and F_2 generations obtained from parental strains C3H and I were more susceptible to spontaneous mammary cancer than those derived from parental strains C3H and C57 Black.

The experiments established the importance of chromosomal factors obtained from the male parent in the development of mammary cancer in mice and indicated that genetic factors control the degree of susceptibility to this neoplasm.

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Effect of Ingestion of Strain C3H Milk in the Production of Mammary Tumors in Strain C3H Mice of Different Ages

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It has been established that the occurrence of mammary cancer in mice is largely dependent upon an influence which is transmitted in the milk of the mother. The incidence of mammary tumors can be reduced (*1*) in high-tumor strains by fostering the young upon low-tumor strain females and can be increased (*2, 3*) in low-tumor strains by fostering the young upon high-tumor strain females.

Following the confirmation (*4*) of Bittner's earlier work (*5*), a series of investigations was outlined to ascertain the nature of the causative agent or agents in the milk of mice of certain strains. The first experiments were designed to determine whether the influence was present in the first milk only or during the entire period of lactation. It was found (6) that it was present in the milk of high-tumor strain females during the entire lactation period. This indicated that milk obtained at any time can be used for experimental purposes.

Since efforts toward the elucidation of the nature of the agent involve the use of animals, experiments were undertaken to obtain a suitable test animal. It was shown (*2, 6*) that if the susceptible young remained with their mothers for the first few days of life, foster nursing by low-tumor females after this period had no influence in lowering the tumor incidence. In addition, it was noted that susceptible young that remained with their mothers for less than 17 hours after birth before being foster nursed by low-tumor females had a 25-percent incidence of tumors, while those remaining with their mothers for less than 24 hours had a 63-percent incidence. This indicated a correlation between the amount of mother's milk the susceptible young ingested and the incidence of tumors.

The 25-percent incidence of tumors in fostered mice that remained with their own mothers for less than 17 hours suggested that some of them may have developed mammary tumors even if they had not obtained any milk from their own mothers. The first experiment described in this report was performed to test this possibility.

The purpose of the second experiment reported here was to ascertain whether 7- or 14-day-old mice are susceptible to the milk influence. For some experiments, such as forced feeding, mice 7 to 14 days of age are much more satisfactory than the newborn.

EXPERIMENTAL PROCEDURE

All mice were raised in this laboratory. Mice of strain C3H were used as the high-tumor strain. The breeding females of this strain (*7*) exhibit a mammary-tumor incidence of 91 percent at an average age of 8.5 months.

Strain C57 Black mice were used as the low-tumor strain. These were the offspring of two litters of mice. One litter was from Bagg (*8*) of the Memorial Hospital, New York. These were representa-

tives of the tumor-resistant line he developed by submitting strain C57 Black mice to forced-breeding experiments. The other litter was procured from Murray (*9*) of the Springville, New York, Biological Station.

Previous studies on foster nursing reported from this laboratory were performed with the descendants of a litter of C57 Black mice obtained from the Roscoe B. Jackson Memorial Laboratory. These animals were satisfactory in the earlier work, but the colony developed an endemic type of pneumonitis which killed practically every mouse when it reached the age of 8 or 10 months. The colony was discontinued, and new colonies were developed from the mice sent by Dr. Bagg and Dr. Murray.

All animals were kept on a diet of Purina dog-chow pellets, with an unlimited supply of water.

The first experiment was begun during June 1939. Some C3H young were obtained by operative removal from the uteri of their mothers, while others were separated from their mothers immediately after birth. All were transferred to C57 Black foster mothers. After the mice were weaned, each female was mated and bore three litters in rapid succession; each litter was killed within 24 hours after birth. Mammary tumors occur in practically all strain C3H females at an average age of 8.5 months if they bear but one litter, but rapid breeding and nonsuckling were introduced in this experiment in order to obtain conclusive results. Bagg (*8*) showed that rapid breeding and nonsuckling increased the incidence of mammary tumors in mice.

There were 28 fostered strain C3H females in the experiment. Thirteen were removed from the uteri of their mothers and lived to an average age of 15.3 months; none developed tumors of the breast. Fifteen were removed from their mothers immediately after birth, and of these, 3 developed mammary-gland carcinoma when 9, 10.5, and 14.5 months of age, respectively; the remaining 12 mice lived to an average age of 15.2 months. The reduction in the incidence of tumors in breeding C3H females from practically 100 to 10 percent shows clearly the pronounced influence of mother's milk upon the occurrence of mammary-gland cancer [1] in this strain of mice.

The second experiment was begun during November 1939 by transferring C3H young within 17 hours after birth to C57 Black foster mothers. The young were permitted to nurse the C57 Black females for 7 or 14 days, when they were transferred back to their own mothers for 3 days.

[1] *There were 31 C3H male mice in the experiment of which 7, or 22.6 percent, developed spontaneous hepatomas. Eighteen of these mice lived to be 15 to 16 months of age when they were killed; 5, or 27.7 percent, had spontaneous hepatomas. The tumors arose in both groups of males. A previous report* (7) *recorded an incidence of 26.87 percent of hepatomas in 370 C3H males and an incidence of 31.2 percent in 170 which were 15 to 16 months of age. It is concluded that foster nursing exerts no demonstrable influence upon the occurrence of spontaneous hepatomas in C3H male mice.*

TABLE 1.—*Results of ingestion of C3H milk for 3 days by strain C3H young mice when 7 to 9 or 14 to 16 days of age, inclusive*

Age at which mice were nursed by own mother	Age at which mice were foster nursed by C57 Blacks	Number of mice	Number developing mammary tumors (in months)										Tumor incidence	Average tumor age
			5	6	7	8	9	10	11	12	13	14		
Days	*Days*												*Percent*	*Months*
7–9	0–6; 10–21	28	1	1	6	4	3	6	3	1	3		100	9.1
14–16	0–13; 17–21	[1] 37		3	2	3	7	5	2	5	2	2	84	9.8

[1] 6 died free from tumor—2 when 10 months old, and 4 when 16 months old.

after which they were again returned to their C57 Black foster mothers. This produced one group of C3H mice which were in contact with, and ingested milk from, C3H female mice when 7 to 9 days of age, and another group which were exposed to C3H milk when 14 to 16 days old. After weaning they were mated and permitted to bear one litter which was killed within 24 hours after birth. The results of the experiment are summarized in table 1. It is seen that the 7-day-old C3H mice were very susceptible to the milk influence if suckled by their mothers for only 3 days. Fourteen-day-old mice were also susceptible, but the results suggest they were more resistant than the week-old mice.

Bittner (*10*) recently reported the occurrence of mammary tumors in a low-tumor subline of strain A mice following ingestion of high-tumor strain A milk. The mice were 4 weeks old and were given milk obtained by squeezing dissected lactating mammary tissue and placing it in feeding dishes.[2] Of 10 mice receiving an average of 1.7 cc. milk, 8 developed tumors and 1 died without tumor; of 10 consuming an average of 0.9 cc. milk, 3 developed tumors. The remaining 8 mice were living and free from tumor when 16 months of age. Bittner's results, together with those presented in this communication, permit the conclusion that mammary-gland tumors can be produced in susceptible mice that are 1 to 4 weeks old provided they ingest sufficient amounts of milk from high-tumor-strain females.

DISCUSSION

The primary object of the two experiments reported here was to obtain information regarding test animals for further work on the causative agent of mammary cancer in mice which is known to be present in the milk of high-tumor-strain females.

The first investigation gave an incidence of mammary tumors of 10 percent in fostered strain C3H females which did not obtain any milk from their own mothers. The relatively low incidence suggests that in previous studies (*2, 6*) the majority of tumors arising in C3H mice which remained with their mothers for from 7 to 24 hours were caused by the ingestion of a small amount of C3H milk. The absence of tumors in 13 fostered C3H mice which were taken from the uteri of their mothers and the occurrence of 3 tumors in 15 fostered mice removed from their mothers immediately after birth cannot be explained at the present time. The result may have been fortuitous, or it may be possible that the process of birth presented an opportunity for a cancer-inciting influence to enter the young. Bittner (*11*) published evidence indicating a wide distribution of the milk influence throughout the body of female mice and that post-uterine contact of the young with the blood of the mother occurs at birth. This question is receiving further consideration.

According to the results of this investigation, operative removal of the young from the uterus is an excellent method for procuring mice which have not been exposed to the milk influence. It is essential to have this assurance in certain experiments, especially those in which progeny are obtained from foster-nursed animals. If the originally fostered mice obtain only a small amount of milk influence, it may be transmitted through several generations of their descendants before it is introduced into a highly susceptible individual and produces a tumor. If the milk influence is capable of increasing in amount, it may require several generations before a sufficient quantity is present to cause tumors.

The occurrence of mammary tumors in

[2] *Bittner, J. J.: Personal communication.*

strain C3H mice which do not obtain milk from their mothers may also be explained by assuming that all tumors of the breast in mice do not arise because of the milk influence. If it is assumed that the milk influence is essential for this type of tumor in all mice, it must be postulated that some enters the young at birth or that the milk of low-tumor-strain females contains some of the agent.

The second experiment revealed that 7- to 14-day-old C3H mice are susceptible to the cancer-inciting influence in C3H milk if the milk is ingested for 3 days. This finding, together with the earlier observation that the influence is present in C3H milk during the entire lactation period, lays the foundation for quantitative studies in which young mice are forcibly fed definite amounts of milk and measured amounts of milk fractions. Such studies are now in progress.

SUMMARY

High-tumor-strain C3H mice were removed from their mothers' uteri and foster nursed by low-tumor-strain C57 Black females. Mammary cancer did not occur in any of 13 female mice which lived to an average age of 15.3 months. Strain C3H females were removed from their mothers immediately after birth and foster nursed by C57 Black females. Mammary tumors arose in 3 of 15 mice, and the remaining 12 lived to an average age of 15.2 months.

Strain C3H mice were foster nursed by strain C57 Black females until they were 7 or 14 days of age and then permitted to nurse their own mothers for 3 days. This procedure led to the production of tumors of the breast in all mice suckled by their mothers when 7 days of age and in 84 percent of those suckled when 14 days old.

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The Relative Activity of Xanthine Dehydrogenase, Catalase, and Amylase in Normal and Cancerous Hepatic Tissues of the Rat

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INTRODUCTION

The desirability of comparing the chemical and physiological properties of a tumor with those of its normal tissue of origin has previously been emphasized (*1*, *2*). At the present time one of the most satisfactory tumors to investigate from this standpoint is the rapidly growing hepatic tumor No. 31 in rats of the Osborne–Mendel strain (*2*). Two factors favor an intensive investigation of the biochemical properties of this tumor: (1) The fact that it arises from the liver cells, thus making direct comparison with normal liver possible; and (2) the possession by liver tissue of a large number of active enzymes whose alterations when the liver cells become malignant can be readily followed.

We described the very low value for the arginase activity of the hepatic tumors of rats and mice as compared with that of the normal liver of these species (*2*). In addition it was pointed out that the livers of rats bearing the transplanted hepatic tumor No. 31 were lower in arginase activity than were the livers of normal animals. The depolymerase for thymonucleic acid was also very much lower in the hepatic tumor than in normal liver (*3*).

In view of these findings, it was considered desirable to extend the investigation to a study of other enzyme systems in these tissues, with particular reference to those enzymes which like arginase appear to be concentrated in the liver. Amylase, catalase, and xanthine dehydrogenase were selected. Comparative data for the hepatic tissues were thereby derived from the behavior of enzymes of both the hydrolytic and of the oxidative variety.

EXPERIMENTAL PROCEDURE

Materials

The rat hepatic tumors employed were transplants of the original tumor No. 31 and were the sixth and seventh transfers (*2*). Tissues used for comparison included the livers of animals bearing the transplant, the livers of normal animals, fetal rabbit livers, and regenerating rat livers at various periods (*2*). Several samples of each kind of tissue were used. Necrotic areas were completely removed. The rats were killed by decapitation. Each kind of tissue was thoroughly ground with quartz sand in a glass mortar immediately after the death of the animal. The paste so produced was taken up in three times the weight of the tissue in distilled water, and the mixture allowed to stand for 18 hours at 5° C. The mixture was then filtered through gauze and the filtrate centrifuged at 1,500 r. p. m. for a few minutes. A determination of total N was then made on an aliquot of the supernatant, and the

latter subsequently adjusted with water to a definite concentration of N.

Extracts so prepared were used as the source of the enzymes investigated. In every case they were used for the determinations immediately after the adjustment to a definite N concentration. For the present studies the activities of xanthine dehydrogenase, catalase, and amylase were followed within a comparatively narrow range of concentration of the extract. That concentration was chosen which yielded a reaction rate which could be followed under our conditions with maximum accuracy. The comparisons of a single enzymic activity for various tissues were made with identical concentrations of the extracts except in the case of the catalase activity of the hepatic tumor. Here the most concentrated extract was necessary in order to observe any activity at all; at the same concentration of a normal liver extract, the rate was too fast to measure. This is, perhaps, an extreme example of the necessity of adjustment of the total N in making comparisons of the enzyme activity of tissues which may vary in such activity. It is usually a matter of several trials before a concentration of extract can be selected at which both strongly active and weakly active tissues will yield results which can be measured with nearly equal accuracy.

The enzymic studies were performed on the tissues from several animals. The variation between individual animals was not greater than 30 percent and was frequently less. The variation between consecutive determinations of the same tissue was not greater than 10 percent. In order to be conservative, however, only differences amounting to 100 percent or more between the activity of the tumor and that of the normal control tissues were regarded as significant.

XANTHINE DEHYDROGENASE

The method of following the activity of xanthine dehydrogenase involved the familiar Thunberg technique. The time necessary to decolorize the methylene blue is inversely proportional to the activity of the enzyme. Two different concentrations of the extract were used for each kind of tissue studied, and from the data in table 1 it is clear that there is a proportionality within this range between concentration and activity.

Inspection of table 1 further reveals that the hepatic tumor is only half as active as normal rat liver. The activity of the livers of normal and of tumor-bearing rats is very nearly the same. The xanthine dehydrogenase activity of fetal rabbit livers is extremely low, whereas that of the normal adult rabbit is distinctly lower than that of the normal rat liver.

The activity of the regenerating liver was low for the first 2 days after operation, but by the third day it had returned to its nor-

TABLE 1.—*Xanthine dehydrogenase activity at 25° C.*

[Test: 1 cc. tissue extract plus 1 cc. M/5 phosphate buffer of pH 6.9 plus 1 cc. xanthine solution (0.0016M) plus 1 cc. methylene blue (0.00124M)]
[Control: Same as test, except 1 cc. water was substituted for the substrate. In every case well over 5 hours required for decolorization.]

Tissue	Total N in extract	Time of decolorization
	Mg. per cc.	*Minutes*
Transplanted rat hepatic tumor No. 31—sixth transfer	2.6	28
	1.3	53
Regenerating rat liver:		
1 day	2.6	27
	1.3	50
2 days	2.6	28
	1.3	56
3 days	2.6	15
	1.3	31
4 days	2.6	16
	1.3	36
6 days	2.6	17
	1.3	35
Normal rat liver	2.6	14
	1.3	37
Liver of rat carrying transplanted hepatic tumor No. 31—seventh transfer	2.6	13
	1.3	35
Fetal rabbit livers (17 days)	2.6	(>300)
Normal adult rabbit livers	2.6	57
	1.3	117

mal level. Controls run on each of the tissues did not reduce methylene blue until well over 5 hours, and hence may be left out of consideration.

Ball showed that the prosthetic group of xanthine dehydrogenase is a flavin (*4*). Kahler and Davis (*5*) found that hepatic tumors are much lower in their flavin content than are normal livers. The lowered xanthine dehydrogenase in hepatic tumors reported in table 1 is therefore quite consistent with the findings of Ball and of Kahler and Davis.

CATALASE

The activity of catalase was measured by the rate of evolution of oxygen from mixtures of the tissue extracts with hydrogen peroxide. For each tissue a plot was drawn of the volume of oxygen against time in seconds. The linear portion of the curve was then used to calculate the rate. The slope of this portion is given in table 2. The apparatus used was similar to that employed by White and Winternitz (*6*).

TABLE 2.—*Catalase activity*

[Temperature 27° C. 1.0 cc. of 29 percent hydrogen peroxide added to mixture of 1.0 cc. extract plus 5 cc. of M/5 phosphate buffer at pH 6.9]

Tissue	Concentration of extract	Rate of oxygen evolution
	Mg. N per cc.	*Cubic centimeters per second*
Transplanted rat hepatic tumor No. 31—seventh transfer	3.0	0.01
	1.5	.007
Regenerating rat liver:		
1 day	.6	1.2
	.3	.3
2 days	.6	1.2
	.3	.3
3 days	.6	1.6
	.3	.5
4 days	.6	1.2
	.3	.3
10 days	.6	1.2
	.3	.3
Normal rat livers	.6	1.7
	.3	.5
Livers of rats bearing transplanted hepatic tumor No. 31—seventh transfer	3.0	1.6
	1.5	.5
	.6	.12
	.3	.05
Livers of rats bearing Jensen sarcoma	.6	.18
	.3	.07
Fetal rabbit livers (17 days)	1.5	.1
Normal adult rabbit liver	1.5	1.0
	.6	.1

Inspection of table 2 reveals the extremely low catalase activity of the hepatic tumor as compared with that of normal rat livers. Fetal rabbit livers are much less active than the normal adult rabbit liver. The latter in turn is less active than the livers of normal rats.

Equally striking is the effect which the transplanted tumor has upon the liver catalase of the animal which bears the tumor. The catalase activity of the livers of such animals is very much below that of the livers of normal rats. The effect produced by the presence of either the hepatic tumor transplant or the Jensen sarcoma transplant is very nearly the same.[1]

There is little if any change in the catalase activity of regenerating liver. The differences observed in table 2 between normal and regenerating livers are probably not significant.

AMYLASE

The activity of amylase was followed by the Willstätter-Schudel technique (*7*). This method employs an iodometric titration to follow the appearance of reducing groups during the digestion. The substrate was glycogen, made up in a 1-percent solution in 1 percent sodium chloride. The procedure was as follows: One cubic centimeter of extract was mixed with 2 cc. of the glycogen solution plus 1 cc. of phosphate buffer at pH 6.9. The mixture was incubated for a definite period of time at 39° C., then set in boiling water for 10 minutes, centrifuged, and the supernatant decanted. The residue was washed with

[1] *Determinations of the catalase activity of similar extracts of the kidneys of normal rats and of rats carrying the transplanted hepatic tumor revealed that the catalase of this organ in the latter animals was two to three times weaker than in the normal animals.*

water, and the washings were added to the main bulk of the solution. Five cubic centimeters of approximately 0.1 N iodine solution was added, followed by 7.5 cc. of 0.1 N KOH. The mixture was allowed to stand for 20 minutes, 1 cc. of 1 NH_2SO_4 was added, and the solution then titrated with 0.02 N thiosulfate. A control containing 2 cc. of water instead of glycogen solution was run simultaneously. The difference in titer of thiosulfate, less by the amount due to the reducing groups of the glycogen originally present, was a measure of the amylase activity inasmuch as the glycogen solution by itself did not yield additional reducing groups during the incubation period. This corrected difference in titer is given in the last column of table 3.

TABLE 3.—*Amylase activity*

[Incubation temperature, 39° C. Data in the last column represent the difference in titer of the mixture with and without added glycogen minus the value due to the original reducing groups of the glycogen]

Tissue	Concentration of extract	Time of incubation	0.02 N thiosulfate
	Mg. N per cc.	*Hours*	*Cubic centimeters*
Transplanted rat hepatic tumors No. 31—sixth transfer	2.6	1	1.0
		2	1.5
		4	2.1
	1.3	1	.5
		2	.9
		4	1.8
Regenerating rat liver: 1 day	2.6	1	.8
		2	1.4
		4	2.3
	1.3	1	.5
		2	.7
		4	1.3
2 days	2.6	1	.8
		2	1.6
		4	2.3
	1.3	1	.4
		2	.9
		4	1.5
4 days	2.6	1	.9
		2	1.4
		4	2.5
6 days	2.6	1	.7
		2	1.5
		4	2.6
Normal rat livers	2.6	1	.7
		2	1.5
		4	2.3
	1.3	1	.5
		2	.9
		4	1.5
Livers of rats carrying transplanted hepatic tumor No. 31—seventh transfer	2.6	1	1.0
		2	1.5
		4	2.5
	1.3	1	.4
		2	.9
		4	1.2
Fetal rabbit livers (17 days)	2.6	1	.5
		2	.8
Normal adult rabbit liver	2.6	1	.7
		2	.9

There is apparently little or no difference in the amylase activity of the hepatic tumor, the liver of normal rats, or the livers of rats bearing the transplanted hepatic tumor. Likewise there is no significant difference between the amylase activity of the fetal rabbit livers and of the normal adult rabbit livers. Similarly, the activity of regenerating liver is similar to that of normal rat liver. The differences in the data noted in table 3 are probably without significance.

Although the amylase activity of the hepatic tumor and that of normal rat liver are practically the same, there is little if any glycogen in the tumor ((*8*) and unpublished observations).

DISCUSSION

From the data presented in this and earlier communications (*2*, *3*), it is clear that the extent of the changes in enzymatic activity of normal rat liver tissue when the latter becomes malignant are quite different for each enzyme. Thus the ratios of enzyme activity of the transplanted rat hepatic tumor to that of normal rat liver for the various enzymes may be expressed approximately as follows: catalase, 1:1,000; arginase, 1:15; thymonucleodepolymerase, 1:1.1; xanthine dehydrogenase, 1:2; and amylase, 1:1. Such differences among the enzymes are striking. When the normal liver becomes cancerous, the various enzymes are apparently affected to different degrees.

It is important, however, to point out that the tumor still bears some imprint of the tissue of its origin, for the xanthine dehydrogenase activity is still fairly strong in the tumor and the amylase activity is equal to that of normal liver. It is possible

that when a normal liver becomes malignant certain enzymes will be more sensitive to such a change than others. Of the enzymes studied, catalase appears to be the most sensitive, while arginase comes next. Xanthine dehydrogenase is changed relatively little, while amylase is apparently not changed at all. It is very interesting to note that this order of "sensitivity" is reflected in the differences in activity of the enzymes in normal rat liver and in the liver of animals bearing the transplanted hepatic tumor. Thus the ratios of enzyme activity of the livers of tumor-bearing rats to those of normal rat liver for the various enzymes are approximately as follows: catalase, 1:10; arginase, 1:2; xanthine dehydrogenase, 1:1; and amylase, 1:1. These results indicate that the presence of the transplanted tumor has a distinct effect on the liver of the animal bearing the tumor and that this effect will be most marked on that enzyme system which is most sensitive to malignant changes of its tissue matrix.

The activity of fetal rabbit livers for all of the enzymes studied, with the exception of amylase, is lower than that of normal adult rabbit liver. The parallelism between the relative activity of fetal to adult liver tissue and that of hepatic tumor to normal liver tissue is striking. A comparison between embryonic and tumor tissue has recently been made by West and Woglom (*9*). With the exception of xanthine dehydrogenase in the early stages of liver regeneration, the enzymic activity of regenerating liver is practically similar to that of normal rat liver. The contrast between the activity of the rapidly growing but controlled growth of regenerating liver and the activity of the rapidly growing but uncontrolled growth of the hepatic tumor is noteworthy and sets the differences found between the normal and the tumor tissues in sharper focus.

SUMMARY

The relative activities of xanthine dehydrogenase, catalase, and amylase have been estimated for transplanted rat hepatic tumors, the livers of tumor-bearing and of normal animals, regenerating livers at varying periods after extirpation of approximately 60 percent of the livers, fetal rabbit livers, and the livers of normal adult rabbits. The results have been compared with those obtained earlier on arginase and thymonucleodepolymerase.

The ratios of activity for each enzyme in the tumor to that in the normal liver vary considerably. This order of variation is followed in the ratios of activity for each enzyme in the livers of rats carrying the transplanted hepatic tumor to that in the livers of normal animals.

Thus the ratios of enzyme activity of the transplanted hepatic tumor to that of normal liver are approximately as follows: catalase, 1:1,000; arginase, 1:15; thymonucleodepolymerase, 1:1.1; xanthine dehydrogenase, 1:2; and amylase, 1:1. The ratios of enzyme activity of the livers of tumor-bearing rats to those of normal rat livers are approximately as follows: catalase, 1:10; arginase, 1:2; xanthine dehydrogenase, 1:1; and amylase, 1:1.

The parallelism between the relative activity of tumor to normal liver and fetal liver to adult liver is pointed out. With the exception of xanthine dehydrogenase in the early stages of regenerating liver, the enzymic activity of the latter tissue is similar to that of normal liver.

We are indebted to Dr. G. B. Mider for samples of regenerating rat liver.

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Effects of Ultraviolet Radiation on Sodium Thymonucleate

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The toxic effect of monochromatic ultraviolet radiation on microorganisms, bacteria, yeast, and fungi shows a maximum of efficiency around 2600A. The wavelengths most effective in producing mutations in cells which are not protected by tissue layers, as in fungous spores (*Trichophyton mentagrophytes*) (*1*, *2*), or liverwort sperm (*3*), are also in the region around 2600A. It is also thought by some investigators that malignant changes produced in the skin by ultraviolet radiation are caused by absorption of the radiation by nuclear constituents (*4*, *5*).

Essential compounds in living cells which have pronounced absorption bands between 2000 and 3000A are proteins and nucleic acids. Most proteins have an absorption band in the 2800A region, and nucleic acids have their most pronounced absorption around 2600A. The nucleic acid absorption is many times the absorption of the proteins in this wavelength range.

The nucleic acid is most condensed in certain bands in the chromosomes and varies during different stages of mitosis as Caspersson (*6*) has shown for *Drosophila*. It probably plays a most important role in the genetical mechanism.

It was thought that some idea about the effect of ultraviolet radiation on living cells could be obtained by irradiating sodium thymonucleate *in vitro*. The effect of the radiation under this condition was followed by measuring changes in the specific physical properties of structural viscosity and streaming birefringence of the nucleic acid.

EXPERIMENTAL TECHNIQUE AND RESULTS

We used as the source of radiation low-pressure mercury-vapor lamps with special glass envelopes. These lamps give about 80 percent of their radiation at 2537A. The residual 20 percent is distributed over the near ultraviolet, visible, and infrared (*7*). Three lamps were arranged symmetrically around the exposure and control cells, as shown in figure 1.

The thymonucleate solution was irradiated in a fused quartz tube and the control in a soft glass tube; both tubes were exposed to the same amount of radiation.

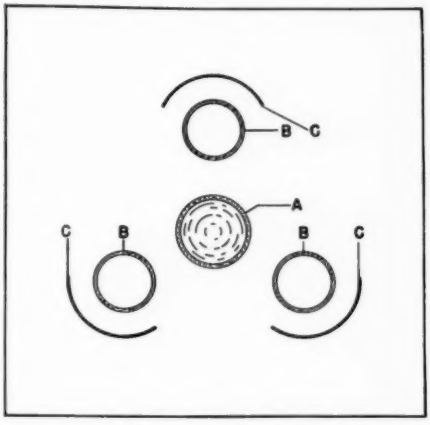

FIGURE 1.—Diagram of irradiation apparatus. *A*, Exposure tube with sodium thymonucleate; *B*, Low-pressure mercury-vapor lamps with glass envelopes; *C*, Reflectors.

The soft glass absorbs all radiation below 3300A. A heavy stream of air was passed over the lamps and tubes, and the temperature was held within 25° to 27° C.

Each tube held about 12 cc.; the time of irradiation was from 4 to 120 hours. The solution was removed for testing every 4 to 24 hours, depending on the number of measurements desired.

Viscosity and Streaming Birefringence

Aqueous solutions of sodium thymonucleate are highly viscous and possess intense double refraction of flow (*8*). The viscosity of these solutions is a function of the velocity gradient at low external pressures (*8*). These phenomena may be interpreted as being due to a high degree of molecular asymmetry of the thymonucleate (*9*).

A 0.5-percent aqueous solution of sodium thymonucleate (part of preparation No. 2 (*8*)) was prepared and divided into two equal parts. One part was placed in a glass tube and allowed to remain under the same conditions as the contents of the irradiated tube. At selected time intervals an aliquot was removed from each tube and the relative viscosity determined at varying external pressures. Simultaneously, the extent of the streaming birefringence was visually estimated. The methods employed for these studies have been described (*8*). The data are shown in figure 2.

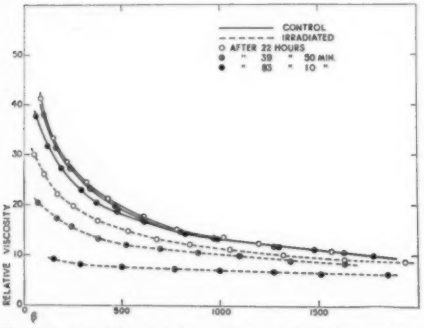

FIGURE 2.—Plot of the relative viscosity against the velocity gradient (β).

The abscissa, β, in the figure is a function of the velocity gradient and was defined in an earlier report (*8*). The dependence of the viscosity values of the nonirradiated thymonucleate solution on the velocity gradient at low values of the latter is shown in figure 2. There was a small but unmistakable decrease in this dependence with standing, owing most likely to a slight spontaneous breakdown of the thymonucleate structure. No such decrease of the viscosity values with time was apparent at the higher velocity gradients, which indicated the very slight extent of the spontaneous changes involved. No significant alteration in the intensity of the streaming birefringence could be estimated visually. On the other hand, the dependence of viscosity on the external pressure was greatly decreased in the case of the irradiated solutions. This dependence furthermore decreased with the time of irradiation, until after about 83 hours the viscosity was practically independent of the velocity gradient. Simultaneously with the decrease in the structural character of the viscosity function for the irradiated solutions, the streaming birefringence also diminished in intensity. After the 83-hour period of irradiation, both properties disappeared. The pH of the irradiated solutions, 6.90, was the same as that of the controls, and free phosphate or ammonia could not be detected within the limit of error of the analyses.

The absorption spectra of sodium thymonucleate before and after irradiation for 83 hours are given in figure 3. The slight variation between these two curves is within the experimental error.[1]

[1] *The absorption spectra were taken by Dr. Peter A. Cole, of the Division of Industrial Hygiene.*

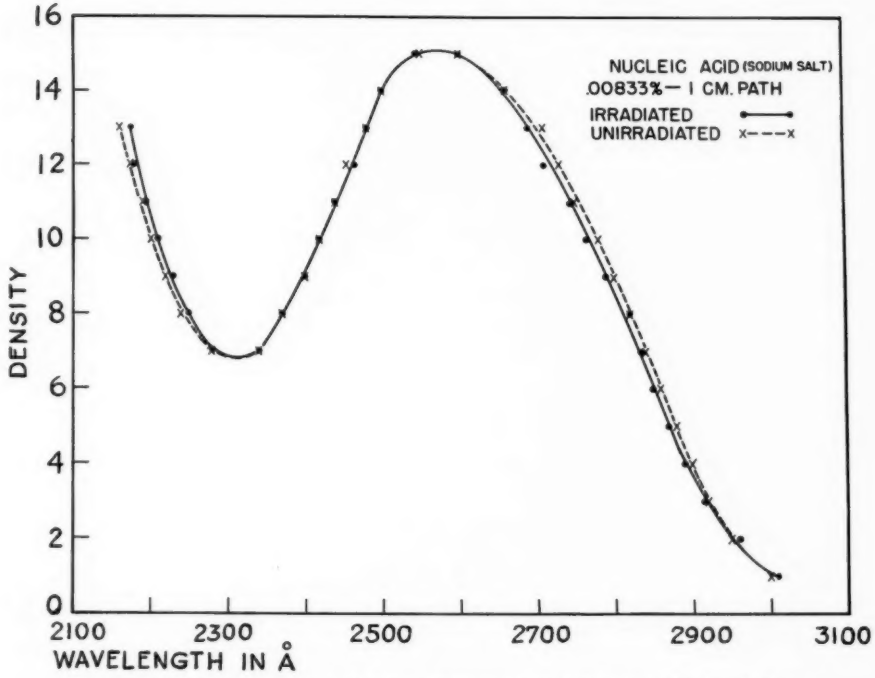

FIGURE 3.—Absorption spectra of sodium thymonucleate before and after irradiation.

The conventional methods of estimating the energy of ultraviolet radiation to which each particle is exposed cannot be applied here on account of the difficulties of stirring, scattering, and absorption. For this reason, we resorted to a method which gives an approximation of the energy to which the sodium thymonucleate was exposed.

Escherichia coli was grown on nutrient agar, washed with physiological salt solution, and added to the sodium thymonucleate solution so that each cubic centimeter contained about 10,000 bacteria. This number of organisms was not so great as to interfere with the radiation process. Samples of 0.1 cc. were removed from exposed and control material at regular intervals during the process of irradiation, diluted with physiological salt solution, and plated out on nutrient agar. At least three plates were poured for each dilution, and usually two dilutions were made. The plates were incubated for 48 hours, and all colonies were counted. Each solid point in figure 4 is the average of at least three plates and is typical of the results obtained. The circles indicate the percentage drop of viscosity at 16 cm. H_2O multiplied by 4. The curves for the decrease of viscosity at 16 cm. H_2O and the survival ratio curve for *E. coli* agree fairly well when this arbitrary constant is used.

Using the data for the incident energy necessary to kill 50 percent of *Escherichia coli* as described by Gates (*10*), and by Hollaender and Claus (*11*), we came to the following calculation: It takes 1.3×10^{-5} ergs per bacterium of absorbed energy at

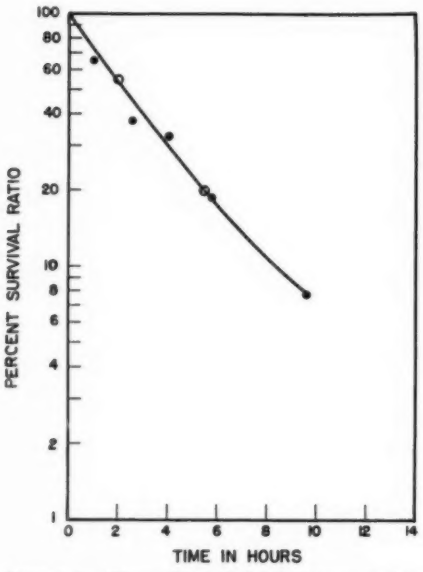

FIGURE 4.—Time of irradiation in hours, against percent of survival ratio for bacteria (●) and the reciprocal of the loss of residual structural viscosity multiplied by 4 (○).

2537A to kill 50 percent of the organisms (*E. coli*) when the bacteria are suspended in physiological salt solution. It took 2 hours of exposure to produce the 50-percent killing when the organisms were suspended in 0.5 percent sodium thymonucleate dissolved in distilled water. *E. coli* has a volume of about $1\mu^3$. In other words, each μ^3 of sodium thymonucleate (0.5-percent solution) absorbed 1.3×10^{-5} ergs. If we accept the value of 1 million for the molecular weight of sodium thymonucleate (*12*), we have:

$6\times10^{17}\times5\times10^{-16}=300$ molecules of sodium thymonucleate per μ^3. Then each molecule has received 4.3×10^{-8} ergs, or

$$\frac{4.3\times10^{-8}}{7.77}\times10^{12}=5.6\times10^{3} \text{ quanta}$$

i. e., it takes 5,600 quanta per molecule to decrease the viscosity at 16 cm. H_2O of 0.5 percent sodium thymonucleate solution by 11.2 percent. These calculations should be accepted with caution, since they include a number of variable factors which may influence the results as follows: (1) Bacteria irradiated in sodium thymonucleate may be more or less sensitive to ultraviolet radiation than are bacteria irradiated in physiological salt solution. However, control bacteria kept in sodium thymonucleate solution without irradiation did not show a higher death rate than when kept in physiological salt solution. (2) Our present knowledge of the molecular weight of sodium thymonucleate is not exact. The value of 1 million is nothing more than an approximation, in view of the fact that sodium thymonucleate in the concentration used in this experiment does not give sharp boundaries in the ultracentrifuge. For this reason we feel that the determination of 5,600 quanta needed to produce 11.2-percent decrease of viscosity in sodium thymonucleate solution should be accepted only as a first approximation and cannot be considered final.

DISCUSSION

The findings that the structural viscosity and the streaming birefringence of sodium thymonucleate decrease with exposure to 2537A radiation may be interpreted as a depolymerization of the highly asymmetric particles into smaller, less asymmetric particles. Such an explanation has already been advanced to account for the loss of these properties in thymonucleate by proteins and salts (*8*) and in myosin by denaturing agents (*13*). The energy of 5.3×10^3 quanta necessary to produce this effect does not seem excessive. Chemical tests and absorption spectra (fig. 3) give no indication of a chemical change in the irradiation solution. The 2537A radiation is absorbed in the purine and pyrimidine ring structures of the nucleic acid mole-

cule, and the energy could be transferred to the bonds which hold the macromolecule together. These bonds in the macromolecule could be broken more easily than the bonds in the micromolecule. The chemical test for free phosphate and the absorption spectra are not sufficiently delicate to pick up the possibly small alterations in the molecular structure which might conceivably occur.

It is interesting to speculate from these findings on the rather complicated processes which are involved in the radiation effects in the ultraviolet, especially from the genetical and physiological points of view. The wavelength range in the neighborhood of 2600A is most effective for lethal action and mutation production. The nucleic acid components of the cell are apparently very readily affected by the ultraviolet. We do not know at present in detail in which condition the nucleic acid is present in the chromatin, but it appears to be partly free and partly in combination with proteins. A break-down of the nucleic acid macromolecule may very well be responsible for changes in the chromosomes which result in mutations. Similar effects of ultraviolet radiation reported here for nucleic acid have been observed by Svedberg and Brohult (*14*) in the case of helix haemocyanin. However, the absorption coefficients for proteins are considerably lower than for nucleic acid.

It would be interesting to obtain data on the effect of other wavelengths between 2000 and 3000A on the depolymerization of sodium thymonucleate. Such studies are in progress and will be reported in a subsequent communication.

SUMMARY

Aqueous solutions of sodium thymonucleate were exposed to radiation, approximately 80 percent of which was 2537A. Measurements of the structural viscosity and streaming birefringence of the irradiated solutions at certain time intervals during exposure revealed a progressive decrease in these properties. After 83 hours of irradiation, a 0.5-percent solution of thymonucleate was completely isotropic. The loss of these properties was interpreted as being due to a progressive depolymerization of the nucleate into smaller, less asymmetric particles.

The energy to which the nucleate was exposed was estimated by the survival ratio of *Escherichia coli* irradiated in thymonucleate solution. From the data obtained, and on the basis of a molecular weight of 1×10^6 for thymonucleic acid, it was estimated that it took approximately 5.6×10^3 quanta per molecule to decrease the viscosity at 16 cm. H_2O of a 0.5-percent nucleate solution by 11.2 percent.

The relation of these findings to genetical mechanisms is briefly discussed.

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The Effect of Pantothenic Acid on Growth and Maintenance of Life in Mice of the C3H Strain

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Pantothenic acid was described and named by Williams, Lyman, Goodyear, Truesdail, and Holaday (*1*) in 1933 as a bios, or essential growth factor for yeast, and its synthesis was reported by Williams and Major (*2*) in 1940. Previously, this component of the vitamin B complex in liver and yeast was separated in the filtrate factor II, of Lepkovsky, Jukes, and Krause (*3*). As such, it was proven to be important in the nutrition of the chick (*4*). Subsequently, pantothenic acid was demonstrated to be an essential nutritional factor in the chick (*4*), rat (*5*), dog (*6*), and recently in the mouse (*7*). For the young rat, with which most of the experimental work has been done, pantothenic acid is necessary for both growth and maintenance of life (*8*).

When the experiments reported herein were started, no information was available on the role of pantothenic acid in the nutrition of the mouse, yet this species is extensively utilized in experimental cancer research. One line of investigation in which the strains of mice having a high incidence of mammary carcinoma have been extremely useful deals with the nutritional requirements of tumor growth. A better understanding of the nutritive requirements of normal mice is needed, however, to give information useful in studying the dietary factors which affect the growth of tumors. At the same time information would be obtained supplementing our present knowledge of the nutritive requirements of the rat, and such information should also give valuable clues to the nutritive requirements in the human. Investigations dealing with the nutritional requirements of the mouse have been neglected in comparison with those made on the rat even though the species are taxonomically closely related.

The neglect of the mouse as an experimental animal in nutritional studies may in part be due to some of the disadvantages of this species for such research. It is known that growth is relatively less rapid in the mouse than in the rat during the short period following weaning in which the maximum rate of growth occurs. Nor does the mouse adapt itself to special diets quite so well as the rat; food spillage is also more frequent and thus it is more difficult to obtain quantitative data. This latter objection can be overcome, however, by utilizing suitable food containers and making the diet nonpowdery.

In taking into consideration the disadvantages of mice for nutritional studies, it is true, nevertheless, that they are adaptable to many types of nutritional investigations. This is particularly true when special synthetic diets are difficult to prepare in large quantities or are expensive. The smaller food intake of the mouse then becomes an important economic consideration.

The C3H strain is especially valuable for tumor-growth studies, not only because of

[1] *Presented before the annual meeting of the American Society of Biological Chemists, Chicago, April 15–19, 1941.*

its exceptionally high incidence of spontaneous mammary tumors but also because of the ease with which it adapts itself to the feeding studies. The experiments reported here have been confined to the C3H strain of mice used by Morris and Lippincott (*9*) in their study of the effect of pantothenic acid on the growth of the spontaneous mammary carcinoma. It should be recognized that the divergent results reported by the few investigators engaged in studying the nutritional requirements of mice may be due in part to strain differences but more likely to some of the disadvantages inherent in mice as experimental animals in this kind of research. It has been found [2] that vitamin supplements which the rat readily consumed when fed separately from the basal diet were not eaten at all well by mice under the same experimental conditions. The fact that a number of investigators (*5, 10–17*) have indicated that the nutritional requirements of the mouse are not identical with those of the rat increases the importance of further nutritional studies with mice.

The use of synthetic diets for growth studies in mice makes possible more precise experiments on the relationship of dietary constituents to normal and tumor growth. In unpublished experiments from this laboratory on the amino acid requirements for growth of young C3H mice, it was observed by Morris that liver extract, Lilly No. 343, contains a substance or substances which induce growth not otherwise obtained. Following the synthesis of pantothenic acid as reported by Williams and Major (*2*), it was thought from further studies that perhaps the inability of mice to grow on a diet in which the protein was supplied by mixtures of amino acids was due to an insufficient amount of this growth-promoting material in the diet. While it has been shown that for promoting growth in the rat β-alanine (*5, 10, 11*) is ineffective in replacing filtrate factor II, now identified at least in part as pantothenic acid, no information regarding this point was available for the mouse.

The present communication deals with the essential requirement of pantothenic acid for life in both young and adult mice, with the effect of the vitamin on the growth of young mice, and with its necessity for the mainenance of body weight in adult mice. Some observations are also reported on the quantitative requirement of this substance for young mice.

EXPERIMENTAL PROCEDURE

The first experiment was designed to show whether or not pantothenic acid is necessary for the growth of young mice. Young male and female mice at weaning time were used for this study. The individual mice usually weighed 9 to 12 gm. at 3½ weeks. There then ensued a period of rapid growth in this strain, the weight reaching 22 to 24 gm. after about 5 weeks. Because the rate of growth declined sharply after that time, subsequent growth studies are of relatively little significance. The animals were kept in individual metabolism-type cages with wire-cloth bottoms in a room maintained at 78° to 80° F. They were fed a synthetic diet, No. 64, composed of Labco vitamin-free casein, 18 percent; sucrose, 53 percent; salt mixture, 6.0 percent (McCollum's No. 185 modified); Crisco, 20 percent; corn oil, 1 percent; cod-liver oil, 1 percent; and wheat-germ oil, 1 percent; without any other vitamin supplementation. When they had ceased to gain in weight and were declining rapidly, the basal diet No. 64 was supplemented by hand feeding (Morris and Thompson (*18*)) of a supple-

[2] *Morris, H. P.: Unpublished work.*

ment consisting of 40γ thiamin, 20γ riboflavin, 20γ pyridoxine hydrochloride, 1 mg. choline hydrochloride, and 1 mg. nicotinic acid per mouse per day. A typical growth curve of one of these animals is shown in figure 1.

This supplementation induced a slight temporary gain in weight, followed by a second decline. At this point 40γ of synthetic calcium pantothenate [3] [4] was added to the supplement. There was an immediate response to pantothenic acid administration resulting in increased body weight approaching almost the normal for this strain. This preliminary experiment indicated the responsiveness of young mice to this vitamin, but no information was as yet available on the optimum amount of pantothenic acid required by young mice. Several investigators had reported pathologic changes occurring in rats from deficiency of pantothenic acid so that it was also considered necessary to establish whether similar pathologic changes developed in both young and adult mice.

For these experiments a new synthetic diet deficient in pantothenic acid was prepared. This diet, No. 80, was made up of the following constituents: Labco vitamin-free casein, 18 percent; sucrose, 55.5 percent; salt mixture (modified McCollum's No. 185), 4.5 percent; Crisco, 20.0 percent; wheat-germ oil, 1.0 percent, and corn oil (Mazola) fortified with vitamin A ester [5] and calciferol [6] equivalent in vitamin A and D content to not less than 3 percent of a U. S. P. XI cod-liver oil. For each gram of basal diet there

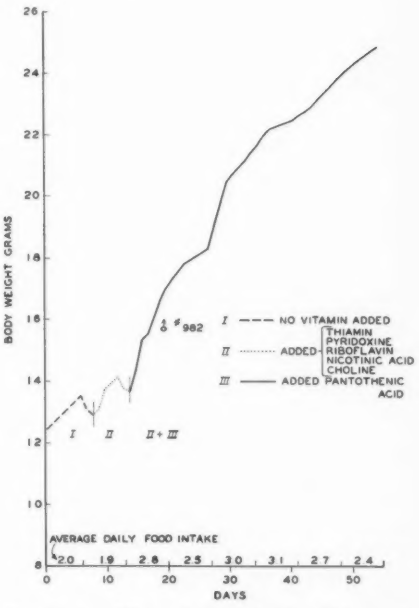

FIGURE 1.—Effect of pantothenic acid on the growth of young normal mice.

were incorporated 40γ thiamin hydrochloride, 20γ riboflavin, 20γ pyridoxine hydrochloride, 500γ nicotinic acid, and 500γ choline hydrochloride.

The deficiency of pantothenic acid in this diet caused a prompt loss in body weight of adult mice, a severe dermatosis, and a definite paralysis of the posterior portion of the body. The effect on body weight of feeding the deficient diet to normal adult virgin C3H female mice is shown in figure 2. These animals were 3 to 4 months of age when the experiments were started and well under the age at which tumors appear in virgin females of this strain. The curve is based on the average weight of 10 mice. There was a striking loss of body weight which began after 1 week on the deficient diet, approximately the same time as when a loss in weight occurred in young mice as shown

[3] *We acknowledge our appreciation to Merck & Co., Rahway, N. J., through the courtesy of Dr. J. M. Carlisle, for generous supplies of synthetic calcium pantothenate used in these experiments.*

[4] *When reference is made in the text to pantothenic acid administration, it refers specifically to the synthetic calcium salt of this acid.*

[5] *Supplied by Distillation Products Co., Rochester, N. Y.*

[6] *Supplied by the Mead Johnson Co., through the courtesy of Dr. C. E. Bills, Evansville, Ind.*

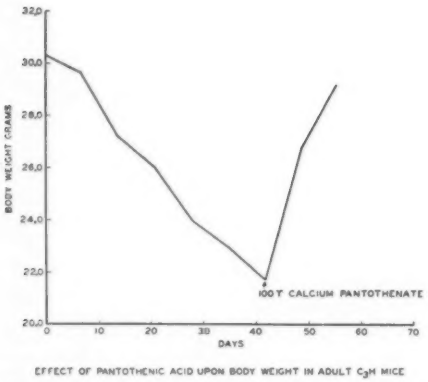

FIGURE 2.—Effect of pantothenic acid upon body weight in adult C3H mice.

in figure 1. When some of the animals appeared near death from the effects of the deficiency, after having lost approximately a third of their initial weight as shown in figure 2, the mice were divided into two groups. Half of them were killed when *in extremis* for pathologic studies. The other half were fed 0.2 cc. daily of an aqueous solution containing 500γ per cubic centimeter of synthetic dextrorotatory calcium pantothenate. There was an immediate response to this treatment as shown by the very rapid recovery of their original body weight. This recovery extended over a period of approximately 2 weeks. Some of the mice that recovered more than regained their initial weight, while others did not quite equal it after recovery. The early loss in weight followed by complete recovery indicates that there is no appreciable storage of pantothenic acid in the body of adult mice and that it is essential for maintenance of body weight.

In view of these effects on growth, a third experiment was devised to secure more information on the quantitative requirements of pantothenic acid for growth of mice. Six groups of 10 animals each were divided uniformly as to sex, breeding, and age. Group 1 received synthetic diet No. 80 without the addition of any pantothenic acid; group 2 received in addition to diet No. 80, 10γ of calcium pantothenate per gram of food; group 3, 20γ; group 4, 40γ; group 5, 80γ; and group 6 was fed diet No. 77 which has been used in this laboratory for several years as an adequate all-purpose stock diet for mice. It is composed of skim-milk powder, 22.75 percent; ground whole wheat, 61.52 percent; dried brewers' yeast, 4.00 percent; cod-liver oil, 2.0 percent; corn oil, 8.2 percent; sodium chloride, 1.4 percent; and ferric citrate, 0.13 percent.

The average growth curves of these six groups of mice are given in figure 3. It will be observed that in no instance did the rate of growth of the animals on the synthetic diets, at any level of supplementation with pantothenic acid, exceed or even quite equal the rate of growth of the animals on the control stock diet. There

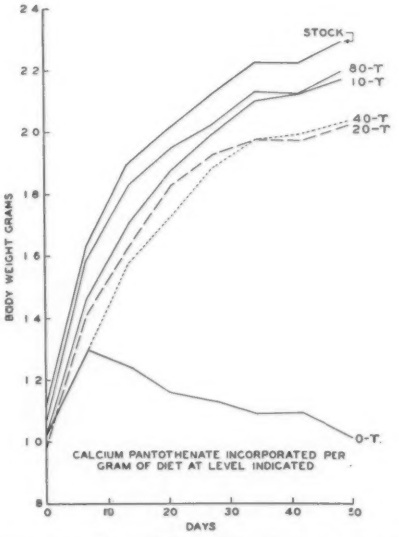

FIGURE 3.—Effect of different levels of pantothenic acid intake on growth of young mice (curves based on average of 10 animals).

was, however, very good growth at all four levels at which the vitamin was fed. If lower levels had been selected, it seems likely that a quantitative response would have been secured. It is quite apparent from the results obtained in group 1, the negative controls (fig. 3), that growth in young C3H mice without pantothenic acid did not extend beyond about 1 week when they were placed on the deficient diet at weaning. Following this slight gain during the first week, there was a very gradual decline in body weight until the death of the animal. This decline was not so great as the loss in weight which occurred in the adult mice.

The pantothenic acid intake of the four groups of mice fed this vitamin is presented in table 1. The animals in group 2 fed at a level of 10γ calcium pantothenate per gram of food as shown in this table, ingested on an average of 23γ to 29γ daily during the 7-week experimental period. At this level of intake they made as good growth as the animals in group 5 which ingested 208γ to 224γ daily. Evidently 23γ to 29γ of pantothenic acid daily is above the minimum requirement for this substance in the mouse under our regimen because there is no indication of growth retardation at this, the lowest level of feeding. The amount ingested is somewhat less than the 30γ reported by Sandza and Cerecedo (7) as the daily requirement for mice. They, however, administered the vitamin subcutaneously. It is apparent, therefore, that calcium pantothenate is as readily available to the animal by absorption from the alimentary tract as from subcutaneous injections.

At about the fourth week on the deficient diet, accompanying the continued loss in weight in both young and adult animals, characteristic changes were noted in the hair. Denuding occurred chiefly on the ventral surface, flanks, and hind legs of the mice. Frequently preceding but occasionally following depilation, a dermatosis developed which presented a scaly, chalklike appearance. The severe dermatosis and loss of hair from the scapular region of the deficient adult animal are shown in figure 4, *A*. Ten days after pantothenic acid therapy was started, the

TABLE 1.—*Average daily calcium pantothenate intake of strain C3H mice*

Group	Body weight, food intake, and pantothenic acid intake	Diet	Level of pantothenic acid, gammas per gram of diet	Week of experiment							
				Initial	1	2	3	4	5	6	7
1	Avg. body wt. (gm.)	80	0	10.0	13.0	12.4	11.6	11.3	10.9	10.9	10.2
	Avg. daily food (gm.)				2.0	1.8	1.5	1.5	1.3	1.3	1.3
	Avg. daily pantothenic acid intake (γ)				0	0	0	0	0	0	0
2	Avg. body wt. (gm.)	80	10	10.1	14.8	17.1	18.8	20.0	21.1	21.3	22.0
	Avg. daily food gm.)				2.3	2.6	2.7	2.8	2.7	2.7	2.9
	Avg. daily pantothenic acid intake (γ)				23	26	27	28	27	27	29
3	Avg. body wt. (gm.)	80	20	9.8	14.1	16.3	18.3	19.3	19.8	19.8	20.3
	Avg. daily food (gm.)				2.4	2.8	3.1	3.0	2.8	2.7	2.2
	Avg. daily pantothenic acid intake (γ).				48	56	62	60	56	54	44
4	Avg. body wt. (gm.)	80	40	10.1	14.1	15.8	17.3	18.9	19.8	20.0	20.4
	Avg. daily food (gm.)				2.4	2.5	2.5	2.7	2.6	2.6	2.4
	Avg. daily pantothenic acid intake (γ).				96	100	100	108	104	104	96
5	Avg. body wt. (gm.)	80	80	10.6	15.9	18.3	19.5	20.3	21.4	21.3	21.8
	Avg. daily food (gm.)				2.6	2.8	2.8	2.8	2.6	2.6	2.6
	Avg. daily pantothenic acid intake (γ).				208	224	224	224	208	208	208
6	Avg. body wt. (gm.)	[1] 77 stock	None added	10.7	16.3	19.1	20.2	21.3	22.3	22.3	23.0
	Avg. daily food (gm.)				3.5	3.5	3.5	3.3	3.4	3.4	3.5

[1] Not isocaloric with diet 80.

hair was growing back, with its normal color, and the skin had apparently recovered from the cutaneous lesion as shown in figure 4, *B*. In these illustrations both sections of the pelts were taken from the same part of the body, and the dermatosis was of about equal severity in both animals before the administration of pantothenic acid to the animal whose skin is shown in figure 4, *B*. The animals fed the synthetic diet plus a daily supplement of 100γ of synthetic calcium pantothenate by stomach tube appeared normal 4 months after recovery from extreme pantothenic acid avitaminosis.

A partial paralysis affecting the hind legs occurred during the latter part of the deficient state in addition to the effects on skin, hair, and body weight. When this condition developed, stimulating the animal by touch resulted in its taking a few stilted steps followed by an intermittent dragging gait and characteristic squatting position involving the posterior third of the body. This condition is partially illustrated in figure 5. A complete cinematographic record of these neurologic manifestations was made. Because of this striking clinical picture a complete study was made of the nervous system. The histologic findings described in detail in a following paper (*19*) demonstrated that myelin degeneration occurs in the spinal cord, posterior roots, and sciatic nerves while the brain remains intact. The adrenals of the deficient

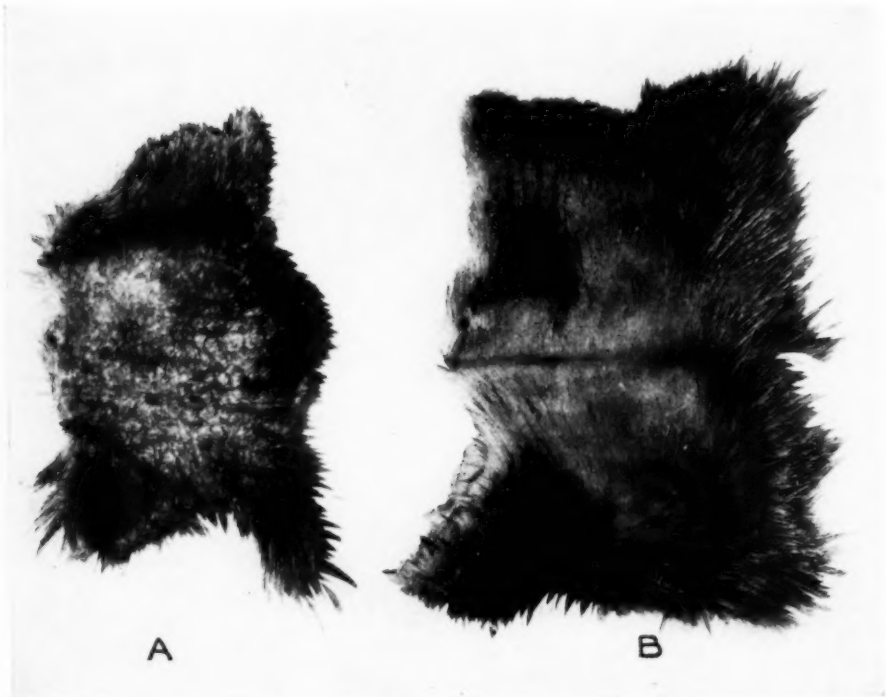

FIGURE 4.—*A*, Severe dermatosis due to pantothenic acid deficiency; *B*, recovery 10 days after pantothenic acid administration.

FIGURE 5.—Characteristic squatting position involving posterior third of body of a pantothenic acid-deficient animal.

animals were normal and identical with those of mice receiving pantothenic acid.

DISCUSSION

The myelin degeneration occurring in the nervous system apparently was not due to thiamin deficiency because each animal consumed more than 1 gm. of food daily. Under these conditions 1 gm. of the diet would contain 40γ of thiamin hydrochloride, which is thought to be entirely adequate for the prevention of pathologic changes in the nervous system of the mouse from thiamin avitaminosis. Further evidence that the animals received an adequate intake of thiamin is furnished by the fact that with only a slight temporary increase in food consumption following pantothenic acid therapy there was, nevertheless, a complete recovery of the mice from the gross symptoms described above.

Hemorrhagic adrenal necrosis in rats on diets deficient in pantothenic acid has been reported in several laboratories (Daft, Sebrell, Babcock, Jr., and Jukes (*8*), Salmon and Engel (*20*), Mills, Shaw, Elvehjem, and Phillips (*21*), and Unna (*5*)).

The fact that these investigators have observed adrenal necrosis in 40 percent or more of their pantothenic acid-deficient animals and that no adrenal changes have been observed in the present work with mice would indicate either one of two possibilities: (1) The diets used by these investigators on the rat were not entirely adequate in some other factor or factors; or (2) there is a species difference between the mouse and the rat in regard to the effect of pantothenic acid upon the adrenal. The mouse usually does not survive on pantothenic acid-deficient diets much beyond 6–8 weeks, a shorter survival period than has been reported for pantothenic acid-deficient rats. It is reasonable to suppose that the shorter survival period of the mouse may account for the fact that no adrenal damage occurs in this animal due to pantothenic acid deficiency and the difference in the species might be explained on that basis. These possibilities need further investigation before it can be definitely concluded or assumed that there is a species difference between the mouse and the rat in respect to adrenal lesions.

Rats apparently do not develop the dermatosis characteristic of pantothenic acid deficiency observed in mice nor do the mice develop, in our hands, ocular exudate, or nose bleed, so frequently observed in pantothenic acid-deficient rats, and only occasionally spectacled eyes.

Certain diets, as described by Norris and Hauschildt (*16*), caused some of the same characteristics as those observed in these experiments. Norris and Hauschildt observed a loss of hair, which, from their description, apparently was more severe than that observed in our experiments. The dandruff-like appearance was much like the skin condition we observed, although the location differed somewhat since it usually appeared first and more severely dorsally in the scapular region. Norris and Hauschildt found it appearing almost simultaneously on the back of the neck and in the inguinal region. They were unable by the addition of the filtrate fraction from either liver or yeast extract to promote growth or prevent the appearance of the skin lesions. The diet used in our experiments differed in some important details from that used by Norris and Hauschildt in containing less sucrose and more fat. They used 1 percent cod-liver oil and 2 percent butterfat, whereas the diet used in our experiments contained 20 percent Crisco, 1 percent corn oil, and 1 percent wheat-germ oil. The latter was used primarily as a source of vitamin E.

In view of the reports of Woolley (*17*) that the mouse requires an antialopecia factor which he (*22*) has identified as inositol or a derivative of inositol, the possibility has been considered that our diet contains traces of this material.

Woolley's (*23*) diet differs from our diet in several other important details; therefore, it seems not possible to evaluate fully the importance of inositol without also considering the much higher levels of the growth vitamins used in our diets. Calculations show that our mice received per gram of food, 20 times more thiamin, 4 times more riboflavin, 10 times more pyridoxine, and 50 times more each of nicotinic acid and choline than did those studied by Woolley. One explanation of the effect of inositol on the cure of alopecia may be related to the low thiamin content of Woolley's diet, for Williams and Saunders (*24*) observed that inositol alone had little effect on the growth of yeasts but in conjunction with vitamin B_1 or pantothenic acid it did increase growth of yeast. Thus, inositol might serve in place of a part of vitamin B_1. The interrelationship of the various growth vitamins is not well understood for the rat, and less well understood for the mouse.

Since we did not get alopecia in our mice when pantothenic acid was added to an otherwise complete diet and since our diet contained much higher levels of the growth vitamins than did Woolley's, and although Woolley cured the alopecia by inositol or its derivatives, it would appear that the low level of the growth vitamins complicates the significance of inositol as an antialopecia factor.

It should be emphasized that no one has secured to date as satisfactory growth with mice as is reported in this investigation with a diet in which the growth vitamins were supplied in the form of chemically pure compounds. The only natural products added of unknown composition were the fats.

SUMMARY AND CONCLUSIONS

1. Young mice grew at nearly the normal rate on a synthetic diet supplemented with thiamin, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, choline, calciferol, vitamin A ester, and wheat-germ oil. The growth was not quite

equal to that on an adequate stock diet, which indicated that there may still be other growth vitamins required by the mouse for normal growth, or else the combination of the constituents fed was improperly balanced in some respect.

2. Pantothenic acid deficiency resulted in failure of growth in young mice and loss in body weight in both young and adult C3H mice. Rapid recovery occurred on administration of the synthetic calcium pantothenate to extremely deficient animals. Death due to the deficiency resulted in both groups in from 8 to 10 weeks.

3. The growth of the young mice was nearly as good when they ingested 23 γ to 29 γ as when they ingested over 200 γ of calcium pantothenate daily. The lowest level of feeding was above their minimum daily requirement.

4. There was no appreciable storage of this vitamin in either young or adult mice as indicated by body-weight changes.

5. A paralysis involving the hind legs and posterior third of the body and a dermatosis in both young and adult mice occurred upon pantothenic acid deficiency. These conditions were completely alleviated clinically by administration of synthetic calcium pantothenate.

Acknowledgment is made to Douglas C. Howard for the technical assistance rendered in conducting these experiments.

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Morphologic Changes Associated With Pantothenic Acid Deficiency in the Mouse[1]

By STUART W. LIPPINCOTT, *research fellow*, and HAROLD P. MORRIS, *senior nutrition chemist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

The striking disturbances in nutrition observed in C3H mice maintained on a diet deficient in pantothenic acid (*1*) led us to study the associated pathologic changes. This has not been done before in the mouse, but has been partially done in the rat in which the following lesions have been recorded: hemorrhagic rhinitis (*2, 3, 5, 7*), spectacled eyes (*2, 3, 5*), nutritional achromotrichia (*2, 3, 4, 5*), cartilage hyperplasia (*2, 5*), abnormal thymic involution (*7*), paralysis (*8*), and adrenal hemorrhagic necrosis (*2, 3, 5, 6, 7*). The last has been considered as the most significant finding, and it has been suggested that partial or even complete adrenal cortical insufficiency might result. Mice under our experimental conditions do not develop any adrenal change. They do, however, show other lesions which are described in this report.

EXPERIMENTAL PROCEDURE

Two groups of C3H mice were studied. The control group, which comprised 20 animals of ages similar to those of the experimental animals, received the following complete diet:

	Grams
Casein (Labco, vitamin-free)	180
Sucrose	555
Salt mixture (185a)	45
Corn oil fortified with vitamin A ester and calciferol	10
Wheat-germ oil	10
Crisco	200
Total	1,000

[1] *Paper presented at the meeting of the American Association of Pathologists and Bacteriologists, New York, April 10, 1941.*

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	Mg.
Thiamin hydrochloride [1]	40
Riboflavin [1]	20
Pyridoxine hydrochloride [1]	20
Nicotinic acid [1]	500
Choline hydrochloride	500
Calcium pantothenate (dextrorotatory) [1]	100

[1] Acknowledgment is made and appreciation expressed for generous amounts of these substances supplied by Merck & Co., through the courtesy of Dr. J. M. Carlisle, medical director.

The synthetic vitamins were added to the basal diet.

The 43 experimental animals received exactly the same diet, save that no synthetic pantothenic acid was incorporated. Food intake and weight curves were kept on each animal of both groups. The experimental animals were subdivided into (1) male and female animals placed on the deficient diet following weaning at the age of 3½ weeks, and (2) male and female adults between 3 and 5 months old, but decidedly before the tumor-bearing age in the case of the female.

The control animals maintained their normal weight and showed no pathologic changes throughout the experiment, which lasted about 6 months. It was found in the experimental group that pantothenic acid was necessary for growth in the young animals and also for life in both young and adult animals. In addition the hair of the animals in this group became thin about the nose, over the scapulae, flanks, and lower abdomen. In these same regions scaling appeared, and the skin felt slightly thickened and occasionally became encrusted about the nose. Apathy appeared. The animals characteristically squatted and dragged their hindquarters

in attempting to move about. When actual walking was undertaken, the hind legs were raised in a stilted fashion with slight arching of the back, and the gait was notably awkward.

A number of the animals on the deficient diet were allowed to proceed to death in order that we might acquaint ourselves with the terminal clinical features. The survival capacity was also thus determined. During the latter phase of the experiment, animals *in extremis* were killed just before death and autopsied.

The fixatives employed were 10 percent formalin, Zenker's fluid, 100 percent ethyl alcohol, Müller's fluid, and a decalcifying solution consisting of lactic and nitric acids. The stains were haematoxylin and eosin, van Gieson's stain for connective tissue, Foot's modification of Bielschowsky's method for reticulum, Verhoeff's elastic tissue stain, Mallory's phosphotungstic acid haematoxylin stain, Best's carmine stain for glycogen, potassium ferrocyanide-hydrochloric acid stain for iron, sudan IV stain for fat, neutral red supravital stain for myelin degeneration, Weil's stain for normal myelin, and Marchi's method for fatty degeneration of the nervous system. In general it was attempted to fix and stain as many of the organs and tissues as feasible according to table 1.

HISTOLOGIC FINDINGS

All tissues and organs were essentially normal except those described specifically.

Adrenal Gland

Because of the attention that has been drawn to the adrenal lesion in the rat, particular note was paid to the adrenal gland which in each of our experimental groups was well within the normal. The morphologic appearance of the adrenals in control animals was not always identical. There was a variation in the size of the zones of the cortex. The zona glomerulosa consisted usually of a fragmentary

TABLE 1.—*Method of fixation and staining of tissues*

Organ	Fixative					Stain										
	10 percent formalin	Zenker	100 percent C_2H_5OH	Decalcifying solution	Müller	Haematoxylin and eosin	van Gieson	Reticulum	Elastic	PTAH [1]	Glycogen	Iron	Fat	Neutral red	Weil	Marchi
Heart	x	x	x			x	x				x		x			
Lung		x				x										
Stomach		x				x										
S. bowel		x				x										
L. bowel		x				x										
Liver	x	x	x			x	x				x	x	x			
Kidney	x	x	x			x					x		x			
Bladder		x				x										
Testes	x	x	x			x					x		x			
Ovary	x	x	x			x					x		x			
Uterus		x				x										
Lymph node		x				x										
Spleen		x				x										
Femur				x		x										
Vertebra				x		x										
Sternum				x		x										
Adrenal	x	x	x			x					x	x	x			
Thyroid	x					x										
Pituitary		x				x				x						
Skin	x	x	x			x	x		x		x		x			
Brain	x				x	x		x		x				x	x	x
Cord	x				x	x		x						x	x	x
Sciatic nerve	x				x	x								x	x	x

[1] Phosphotungstic acid haematoxylin.

outer fringe of cells. The zona reticularis was much more variable in depth and was as much as many cell layers thick. The zona fasiculata constituted the major portion of the cortex, and the difference in tinctorial quality and granularity of the cytoplasm was notable. Therefore, tinctorial alteration in cytoplasm may not in itself necessarily indicate a lesion. The medullary cells in the normal animal may have at times a light staining cytoplasm, and their intervening sinusoids are prominent. In the experimental animals such customary features of degeneration and necrosis as pycnosis, karyorrhexis, and the crumbling of cells into debris were entirely absent. Sudanophilic material was abundant in the cells of the zona fasiculata. Glycogen was absent except for small particulate deposits in the cytoplasm of widely separated cortical cells. Stains for iron were negative. van Gieson stains for connective tissue showed no focal areas of fibrosis. Congestion of sinusoids and hemorrhage was never seen in the corticomedullary region. Summarily, it may be said that the adrenals in the experimental group were in no way different from those of the control animals (fig. 1, *A* and *B*).

Osseous System

The nutritional effect upon growth in our mice and the reported hyperplasia of cartilage in the rat make the study of the bones interesting. Likewise the marrow is of interest for panmyelophthisis was described in rats with diets deficient in the vitamin B complex in the investigations of György, Goldblatt, Miller, and Fulton (*9*). The epiphyseal line in the femur of our animals, however, appeared normal. Though the young animals grew more slowly, there was no evidence of hyperplasia or atrophy of cartilage and no morphologic evidence of abnormal ossification. The marrow was never agranulocytic or aplastic (fig. 2).

Heart, Liver, and Kidney

In an occasional experimental animal the cytoplasm of isolated myocardial fibers contained sudanophilic material. Also in a few livers, small and large red droplets,

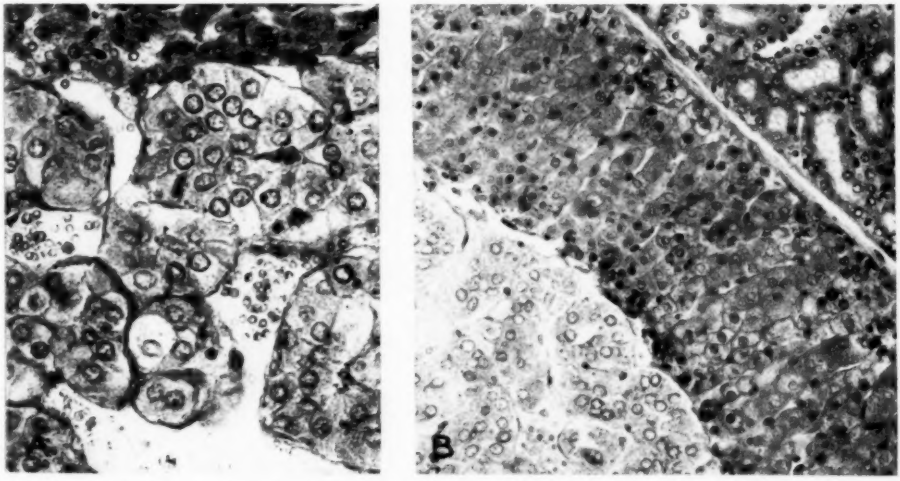


Figure 1.—Normal adrenal from deficient adult mouse. *A*, ×200; *B*, ×350.

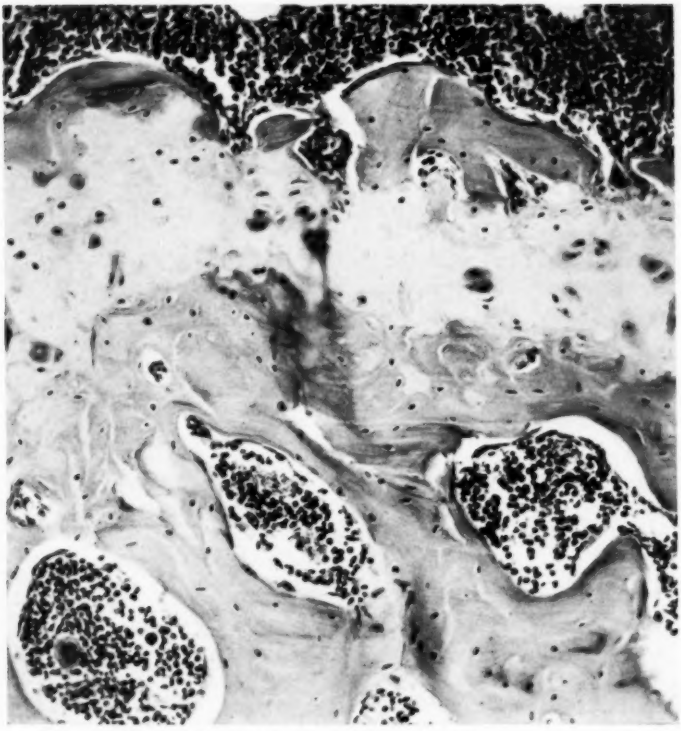


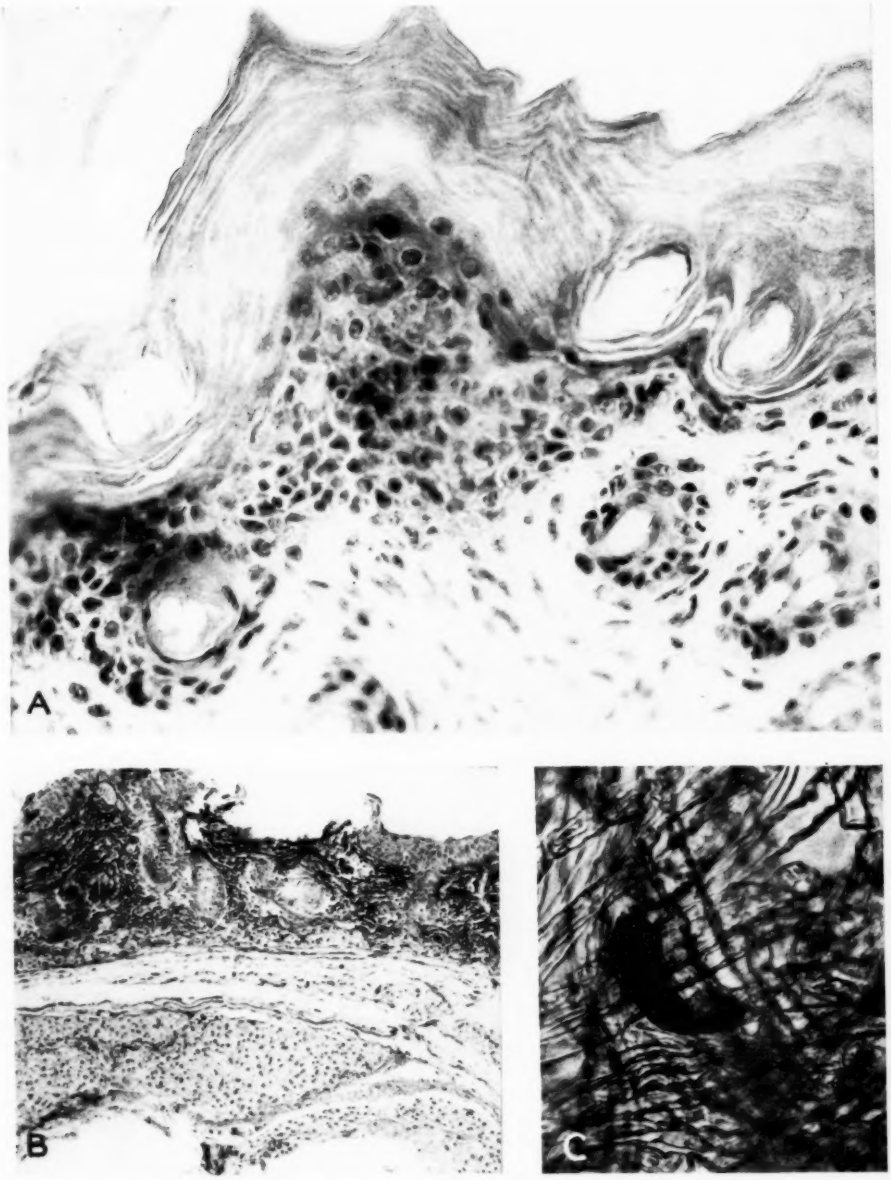
FIGURE 2.—Normal femur. ×250.

with the sudan IV stain, were apparent in the cytoplasm of about 2 percent of the hepatic cells. In a few renal sections, sudanophilic material was present in tubular epithelium and sufficiently widespread to be characteristic of fatty degeneration.

SKIN

Sections taken through the areas of depilation and scaliness showed degenerative but not inflammatory lesions. From these regions stretches of epidermis appeared normal, but more frequently slight to moderate hyperkeratosis was present (pl. 1, *A*). Engulfed in the keratotic layers adjacent to the stratum germinativum were poorly staining, indistinct, squamous cells. As these cells became squeezed together, they clumped into unrecognizable debris. The latter was negative for peroxidase granules. Beneath the hyperkeratotic segments the cells of the malpighian layer became flattened or disappeared. However, without overlying hyperkeratosis, atrophy and complete disappearance of epithelium occurred. Erosions developed slowly, with the base of the defect formed by the normal collagen fibers of the corium. This is seen in a section from the region about the nose (pl. 1, *B*). Epithelial hyperplasia did not occur. The process appeared to be a hyperkeratotic, atrophic and desquamative dermatosis.

PLATE 1



A, Hyperkeratosis of skin overlying scapulae. × 385; *B*, Erosion in epithelium from region about the nose. × 85; *C*, Myelin degeneration in teased preparation of sciatic nerve. × 580.

Nervous System

The sciatic nerve, brain, and spinal cord were examined.

Freshly teased preparations of sciatic nerve were stained with neutral red according to the technique of Covell (*10*). With this method, myelin degeneration is presumably recognized by a homogeneous, waxy, light orange-red accumulation of the dyestuff in the region of the sheath and enclosed axis cylinder. In the young experimental animals such formations were not observed, but they were consistently found in scattered sheaths in the adults (pl. 1, *C*). Sciatic nerves and their branches (pl. 2, *A*) from the same group of adults were stained by the Marchi method. Here again numerous individual sheaths are singled out by sharp, circumscribed, black droplets. These droplets were rarely found in the sciatic nerves of control animals and when present in the latter were peripherally placed and conceivably due to trauma at autopsy.

Sagittal and coronal sections of the brain were made. Neurones appeared intact, and no myelin degeneration was in evidence. In the cord, cervical, thoracic, and lumbar levels were studied. In the young animals of the control group the Weil technique (*11*) indicated that the full complement of myelin was not present, so that in the experimental animals small gaping areas disclosing lack of myelin did not indicate that degeneration was present. With the Marchi stain these same animals showed numerous foci of peripherally placed black droplets in the posterior funiculus. It is possible, though, that some of these may be so-called Elzholz bodies (*12*) considered as artifacts by many. In the adult group of experimental animals the Marchi stain showed rather heavy concentrations of distinct black droplets in the posterior funiculus (pl. 2, *B*). They occurred in the fasiculus gracilis, fasiculus cuneatus, and the region of the pyramidal tracts. The posterior roots were also involved. In the control animals black droplets occurred but infrequently and then were widely dispersed. This finding in the cord is compatible with what is usually accepted as morphologic evidence of myelin degeneration.

While it appears on morphologic grounds as though the skin with its dermatosis and the nervous system with its myelin degeneration were more strikingly affected than any other tissues or systems, these lesions are more properly to be considered as demonstrable expressions of the general disturbance in tissue metabolism. This basic alteration in metabolism is attested by the inability of the animals to survive in the complete absence of pantothenic acid in the diet. Therefore, the deficiency affects all of the tissues from a biochemical viewpoint, but only a few tissues give morphologic indication that lesions are present.

SUMMARY

Histopathological studies were made of young and adult C3H mice maintained on a diet deficient in pantothenic acid until death intervened in 6 to 8 weeks.

1. Unlike those of the rat, the adrenals of the mice remained normal, and the mice did not develop adrenal hemorrhagic necrosis on this diet.

2. A hyperkeratotic, atrophic, and desquamative dermatosis developed.

3. Myelin degeneration was found in the sciatic nerves and in the spinal cord, chiefly in the fasiculus gracilis, fasiculus cuneatus, the region of the pyramidal tracts, and posterior roots.

Acknowledgment is made to Albert Kessel and Thomas Digges for their technical assistance in carrying out this experimental study.

PLATE 2

A, Myelin degeneration in sciatic nerve. × 100; *B*, Myelin degeneration in spinal cord. × 280.

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Effect of Pantothenic Acid on Growth of the Spontaneous Mammary Carcinoma in Female C3H Mice

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The problem of the basic nutritional requirements for tumor growth remains unsolved. However, various experimental approaches have been made to determine the factors involved. The chief obstacle is our limited knowledge of all the chemical constituents essential for normal growth and tissue metabolism in animals. Since the present methods for nutritional studies *in vitro* are not adequate, it is preferable to confine nutritional experiments in this particular field of tumor metabolism to animals in whom spontaneous malignant tumors develop with regularity.

The growth of a spontaneous tumor bears a relationship to the metabolism of the host. Therefore a factor affecting the host's nutrition might also conceivably affect that of the tumor. This is not only a point of pure academic interest but might be of practical significance as an adjunct in tumor therapy. This would be so, for example, if it could be shown that the removal of some substance from the diet limited the rate of growth of the tumor materially without affecting the host's nutrition too adversely. Furthermore, it is also desirable to establish whether or not normal and neoplastic tissues have in their metabolism similar demands upon specific food substances. A knowledge of the nutritional requirements of host and spontaneous tumor are, therefore, of importance.

Several investigators have proceeded in part along these lines. Voegtlin and associates (*1*, *2*) were among the pioneers in this field. In their first experiments they fed a diet composed of 70 percent ground wheat and 30 percent whole-milk powder to mice with spontaneous mammary carcinoma. This resulted in rapid tumor growth, but simple heating of the milk powder supplied with the diet resulted in inhibiting the rate of tumor growth. This inhibition of neoplastic growth could be overcome by administration of the essential amino acid, lysine. Similarly, protein fed in the form of gliadin which is deficient in lysine inhibits tumor growth. Restoration of growth was accomplished by adding the amino acid, lysine, directly or indirectly in the form of glutenin. Later experiments showed that the growth of spontaneous mammary carcinoma could be inhibited by a diet deficient in cystine and methionine (*3*, *4*, *5*). All of these experiments were indications that the rate of growth of these spontaneous mammary tumors could be inhibited by a deficiency of certain essential amino acids.

Bischoff and Long (*6*) made another contribution by showing that caloric restriction per se, without deficiency of specific nutrients essential for growth, resulted in growth retardation of sarcoma 180. In contrast Tannenbaum (*7*) found that the rate of growth of spontaneous tumors developing in animals fed insufficient quantities of a normal diet appeared to be the same as that of tumors developing in full-fed animals. Subsequently Bischoff and Long (*8*) in their studies on the relation

of vitamin B complex to tumor growth tentatively concluded that vitamin B_6 and the filtrate factor were essential for growth of sarcoma 180. It has been reported, on the other hand, that in certain cases some essential dietary constituents do not affect tumor growth. Thus Bryan and Mason (*9*) suggest that vitamin E may be essential for "maintenance of nuclear structure and function in rapidly proliferating tissues." However, they state that a dietary deficiency in this substance had no effect whatever on several successive transplantations of the Walker 256 mammary carcinoma.

These past experiments have shown that amino acids are of importance in the actual formation of tissues whether normal or neoplastic. Likewise the consideration of total caloric intake in its anabolic action falls into the same category. These are expressions of food substances acting as materials which go into the formation of tissues as sources of energy or as the building stones. Although vitamins, such as pantothenic acid, can be detected in minute traces in tissues, their function is probably regulatory and they may not necessarily be integral structural components of tissues. Pantothenic acid is known to be essential to life in several species and may act as a regulatory influence in normal animals as regards growth and maintenance of body weight. For that reason it appeared that pantothenic acid as a growth-promoting substance might have a significant function in tumor metabolism, and it was therefore selected for use in our experiment.

EXPERIMENTAL PROCEDURE

It was thought best first to establish the standard rate of tumor growth in an animal on a full diet by measuring with calipers the size of the tumor at definite intervals from the time it was detected until the death of the animal. This rate was then compared with that of the animals on a similar diet lacking only the specific component, pantothenic acid, or conversely containing an overly abundant amount of this vitamin. The rate of tumor growth, by using statistically significant numbers of animals, then serves to indicate the relationship of the necessity of this dietary substance to *in vivo* tumor metabolism and simultaneously it can be determined whether or not the animal's nutrition is such that the animal has a reasonable survival rate.

Female strain C3H mice were used throughout this experiment. All spontaneous mammary tumors occurring in this strain are considered as primary neoplasms so the individual animal may have from one to several primary tumors. As soon as the spontaneous mammary tumors became palpable, they were biopsied. In all, 55 animals were used. Following closure and healing of the wound at about 4 to 5 days after biopsy, the animals were placed on the experimental diet in individual metabolism cages. At death all but a few of these animals were autopsied, and tissue was taken from the original tumor. Female mice of the C3H strain prior to the tumor-bearing age have the characteristic breast duct and acinar pattern described by Taylor and Waltman (*10*) for other high-strain, mammary-tumor-bearing mice. As indicated in figure 1, *A* and *B*, there are many branching primary and secondary ducts with multiple groups of acini. Morphologically, the tumors observed at biopsy were either adenocarcinomas, which in some areas appeared as carcinoma solidum (pl. 1, *A*), or cystadenocarcinomas (pl. 1, *B*). Microscopic section from the tumors taken at autopsy showed maintenance of the same types of tumors as seen in the biopsies, and therefore the dietary pro-

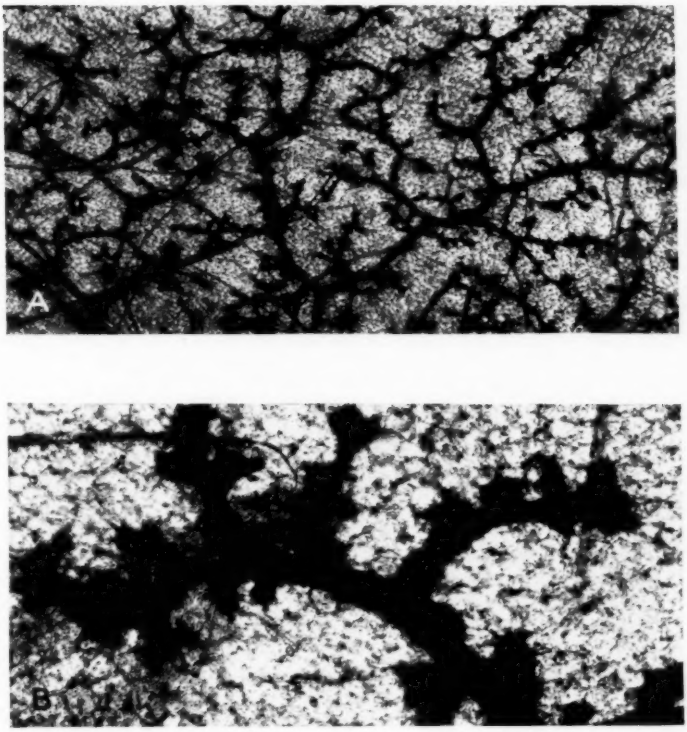
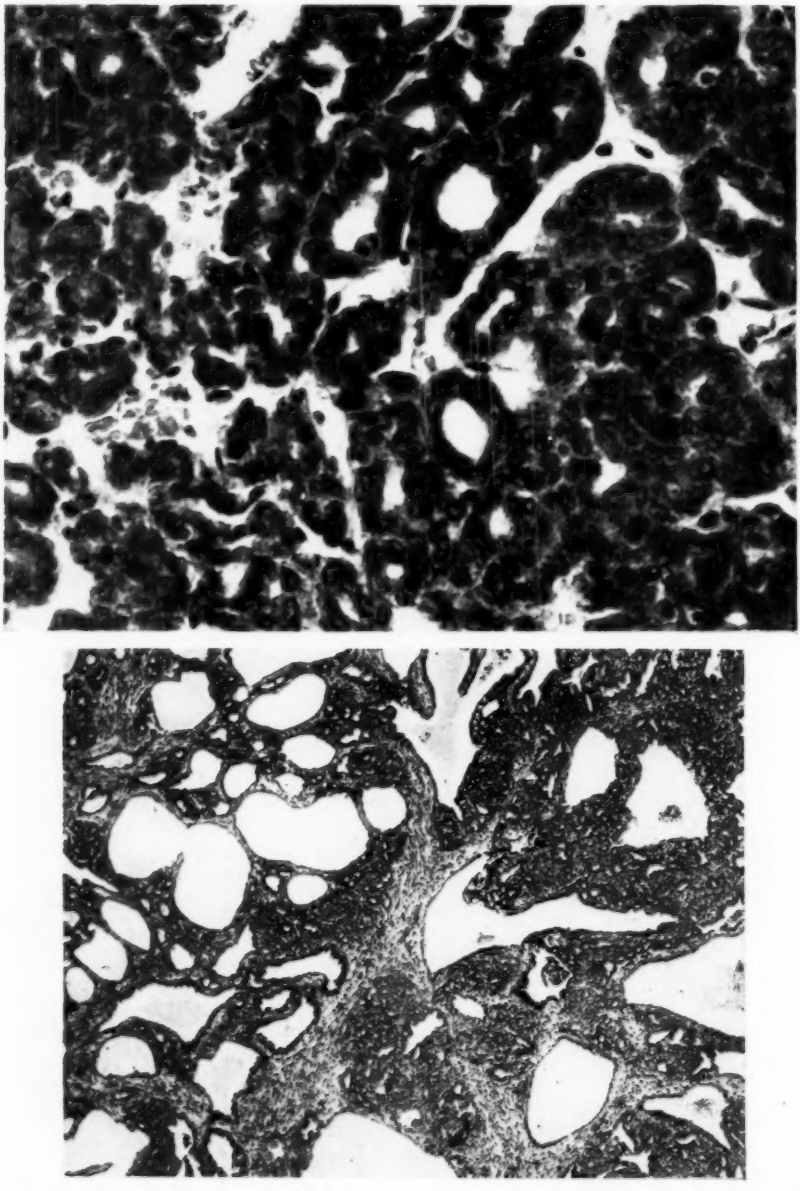


FIGURE 1.—Duct and acinar portion of C3H female breast prior to tumor development. A, X 16; B, X 55.

cedure had no effect upon the morphologic character of the tumors.

In determining the rate of growth of these tumors the two largest dimensions of the tumors were measured by means of a 2-inch caliper. The tumor size was then calculated by obtaining the product in millimeters of the two observed measurements. This is essentially the method used in this laboratory for some time for obtaining the size and growth rate of each individual tumor. When an animal had more than one tumor at the time it was entered in an experiment, each tumor was measured and considered separately. Thus the number of tumors may be, and usually is, larger in any given group than the number of animals in that group. When new tumors appeared they were noted and measured; while they did not appear to affect the growth rate of the original tumors, they were not included in the curves as shown in figure 2. Because such a procedure would decrease the calculated average rate of growth of the first tumor inasmuch as the additional tumors would always be small when they were first observed and if included in the average value plotted in figure 2, they would depress the calculated average growth rate of these tumors. The figures in the circles represent the number of

PLATE 1



A, Adenocarcinoma of breast in a female C3H mouse. × 465; *B*, Cystadenocarcinoma of breast in a female C3H mouse. × 110.

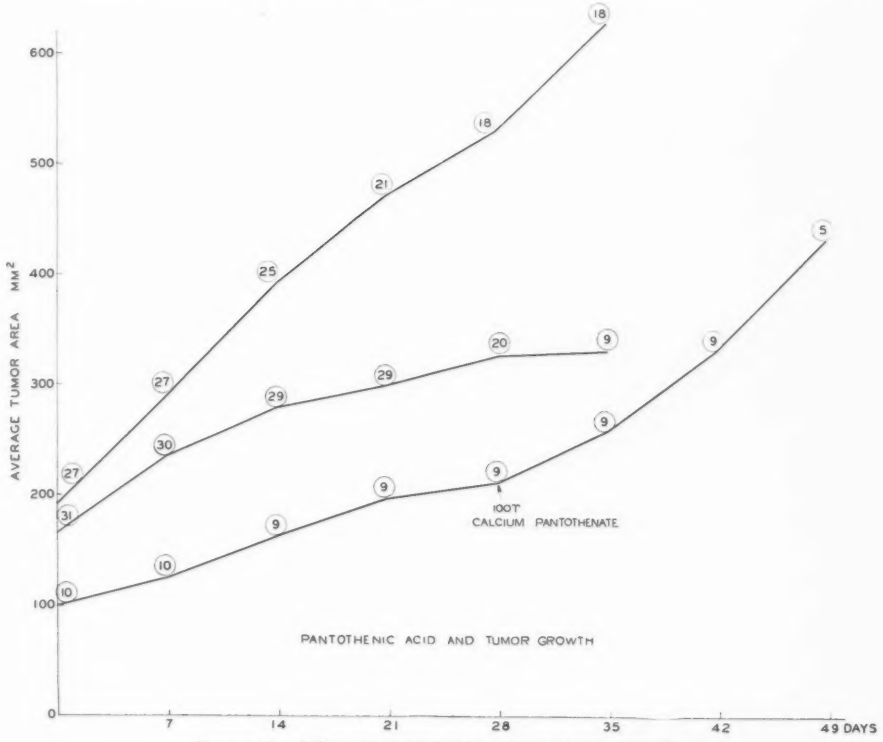


FIGURE 2.—Effect of pantothenic acid on tumor growth.

spontaneous tumors included each time averages were obtained from which these curves were plotted.

The animals used in these experiments were divided as follows: One group was placed on a complete synthetic diet, No. 79. In this diet all the growth vitamins were added in the form of chemically pure components. The second and third groups were placed on diet No. 80 which is identical with diet 79 except that pantothenic acid was omitted. The second group remained throughout on diet 80, while the third group, after 5 weeks on the deficient diet, were fed in addition to the basal diet 100γ daily of calcium pantothenate. The vitamin was administered in a volume of 0.2 cc. through a stomach tube by the procedure of Morris and Thompson (*11*). The constituents of these synthetic diets have been described according to the above diet numbers by Morris and Lippincott (*12*).

By using this procedure, the rate of growth of the tumors for the mice on diet No. 79 represented the control group, and the rate of growth on diet No. 80 represented the findings in pantothenic acid-deficient animals. The group on the complete synthetic diet received considerably more pantothenic acid than the group of hand-fed animals. The amount received in the latter group is much above the requirement shown by Morris and Lippincott (*12*) to produce adequate growth in normal young mice.

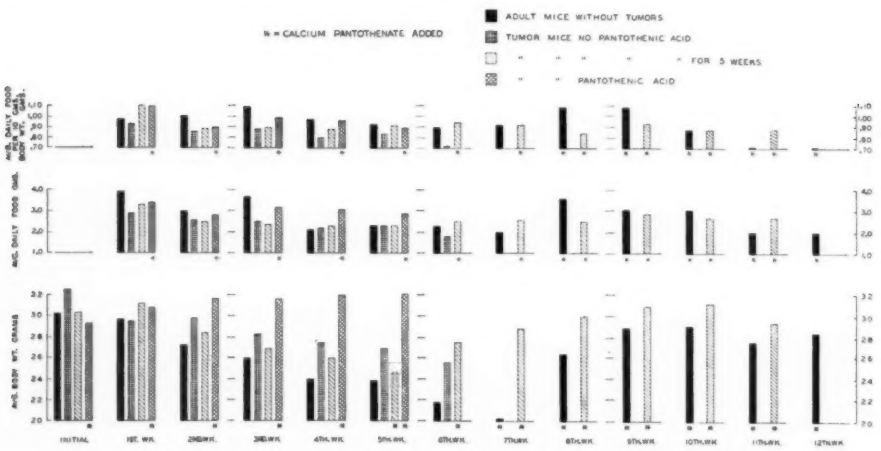


FIGURE 3.—Body weight and food consumption of mice with and without tumors.

It will be observed that the results presented by the upper line of figure 2 are essentially linear and represent the average growth rate of the spontaneous tumors in the animals on the control diet. This tumor-growth rate in these experiments was much more rapid than the rate of growth in either group on the pantothenic acid-deficient diet as shown by the middle line and the first portion of the lower line in figure 2. When the vitamin was fed to the group which had been on the deficient diet for 5 weeks, as shown by the terminal half of the lower curve of figure 2, the rate of growth of the tumors was markedly stimulated, and after the first week of supplementation the growth rate appeared essentially the same as that of the control group. The rate of growth of the tumors in both groups receiving the diet deficient in pantothenic acid decreased with time, which indicated that as the deficiency became more severe it was reflected in the decreased rate of growth of the tumors.

In the case of a great many deficient diets the animals progressively eat less food, and the decreased food or caloric intake per se may be an important factor in limiting tumor growth. In figure 3 are presented the average body weight of adult female mice with and without tumors on diet No. 80, the average weights of tumor animals on complete diet No. 79, and the effect of addition of pantothenic acid to the deficient diet of tumor and normal adult mice. Figure 3 also gives average daily food consumed in the various groups and the average food intake per 10 gm. of body weight.

It will be observed that there was a progressive decrease in body weight on the pantothenic acid-deficient diet in both normal and tumor-bearing adult mice. There was no decrease in body weight in the group of animals eating the complete diet. The animals were given access to these diets ad libitum. When pantothenic acid administration was instituted, as shown by * in figure 3, the animals regained their lost weight in about 14 days. There was an appreciable decrease in total food intake over the deficient period and a less marked increase in food intake in the tumor group during the period when pantothenic acid was present and

the animals were regaining their initial weight. If this average daily food consumption is calculated on the basis of unit body weight of the animals (10 gm. used as the unit in order to keep the calculated values near to unity), it is seen that there is practically no consistent difference in food intake. This is so, even in those cases where the animals were gaining weight very rapidly. On the basis of these food-intake records, it appears that there has not been sufficient decrease in food intake on the average to have affected the growth of these tumors because of decreased caloric intake.

SUMMARY AND CONCLUSIONS

1. Female C3H mice bearing spontaneous mammary carcinomas were fed a diet deficient in pantothenic acid. The rate of growth of spontaneous mammary carcinoma in these mice was markedly retarded when compared with the rate of growth of similar tumors in animals receiving calcium pantothenate. Conversely, when calcium pantothenate was given per os to tumor-bearing mice after a 5-week deficiency period, the rate of growth of the tumors returned to approximately that in the normal control group. As the period lengthened in which the host was maintained on the diet deficient in pantothenic acid, there was a greater decrease in the rate of tumor growth.

2. The average daily food intake of the animals having tumors was very little less before than after pantothenic acid administration. It is believed that the difference in food intake did not account for the marked retardation of tumor growth observed in the deficient animals, for when the food intake is calculated per unit of body weight the food consumption of the deficient animals was essentially the same or in some cases even exceeded the intake of the animals receiving pantothenic acid.

3. Both the normal and neoplastic tissues of these animals require pantothenic acid in their metabolism, although quantitatively the amount may not be the same.

4. In these experiments retardation of tumor growth by a diet deficient in pantothenic acid results simultaneously in such severe interference in the host's nutrition that the procedure offers no practical application in adjunct tumor therapy.

Acknowledgement is made to Douglas C. Howard for technical assistance rendered in conducting these experiments.

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Toxic and Carcinogenic Effects of Stilbestrol in Strain C3H Male Mice

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The effects of long-continued subcutaneous injections of stilbestrol (4,4′-dihydroxy-α, β-diethylstilbene) in mice of strain C3H were described in a previous report (*1*). The present communication is a continuation of the studies, with particular reference to the oral route of administration and to the effects of toxic doses of the compound.

EXPERIMENTAL PROCEDURE

Male mice of strain C3H, raised at the National Cancer Institute by Dr. H. B. Andervont, who has recently recorded the occurrence of spontaneous neoplasms in this stock (*2*), were used in these experiments. The animals were from 2 to 3 months of age at the beginning of the experiment. They were maintained in wooden boxes. The diet, devised by Dr. H. P. Morris, contained graham flour, 61.52 percent; milk powder, 22.75 percent; corn oil, 8.2 percent; brewers' yeast, 4 percent; cod-liver oil, 2 percent; sodium chloride, 1.4 percent; and iron citrate, 0.13 percent; the supply of water was unlimited.

Stilbestrol[1] was dissolved in sesame oil, the amount of solvent being limited to 0.1 cc. per administration.

Experiment 1

Forty-one C3H males, weighing 21 to 23 gm., were used to ascertain the effect of stilbestrol given by the oral route.[2] Stilbestrol in sesame oil was fed to the mice by means of a blunted curved metal tube passed into the esophagus (*4*). The animals were divided into four groups: group 1 (11 mice) was given 0.125 mg. of stilbestrol in 0.1 cc. of sesame oil twice a week for 17 weeks, for a total of 4.25 mg.; group 2 (10 mice) received 0.375 mg. of the compound in 0.1 cc. of sesame oil twice a week for 17 weeks, for a total of 12.75 mg.; group 3 (10 mice) was fed 0.1 cc. of sesame oil twice a week for 17 weeks; and group 4 (10 mice) was kept as untreated controls. The animals were examined and weighed once a week.

Experiment 2

As soon as it became apparent in experiment 1 that there was a marked retardation in gain in weight in the animals which were ingesting stilbestrol, another series was started in order to ascertain whether this effect was due to decreased food consumption. Fifteen C3H male mice, kept in individual cages, were divided into three groups of five mice each: group 1 received 0.375 mg. of stilbestrol in 0.1 cc. of sesame oil twice a week; group 2 received 0.75 mg. of the compound biweekly; and group 3 received 0.1 cc. of sesame oil twice a week.

The mice were weighed and the food intake was measured once a week for 8 weeks. The high mortality among the animals of the first experiment, beginning with the eleventh week, led to reduction of the stilbestrol dose in these groups to 0.2 mg. twice a week after the first 15 administrations (7.5) weeks. Thus, the mice of the first group received a total of 8.625 mg. in 15 weeks, and those in the second group, 14.25 mg.

[1] *Furnished by Merck & Co.*

[2] *A preliminary report on this group has been published* (3).

EXPERIMENT 3

To compare the effect of the subcutaneous and the oral route of administration of stilbestrol, 40 C3H males, 3 months old, were divided into four groups: 10 mice in group 1 received subcutaneously twice a week 0.125 mg. of stilbestrol dissolved in 0.1 cc. of sesame oil; 10 in group 2 received 0.25 mg. of the compound twice a week; 10 in group 3 received 0.5 mg. of stilbestrol twice a week; and the 10 in group 4 were given 0.1 cc. of sesame oil twice a week as controls.

Five animals were kept in each cage, and individual weights and the total food consumption were determined once a week. Since the induction of mammary tumors in C3H mice following subcutaneous injections of stilbestrol was reported previously (*1*), the surviving mice were killed at the end of 4 months.

RESULTS

Stilbestrol was more toxic to strain C3H males when administered by subcutaneous injection than when given by mouth. With biweekly oral administrations, no mortality was encountered until the eleventh week with doses up to 0.75 mg. As indicated in figure 1, 5 of 10 mice died in 11 to 16 weeks after biweekly doses of 0.375 mg., and 4 of 11 died in 17 weeks after 0.125-mg. administrations. In the second experiment, only 1 animal that received 0.75 mg. of stilbestrol died 15 weeks after the initial ingestion; in these groups, however, the dose was reduced to 0.2 mg. after 7.5 weeks.

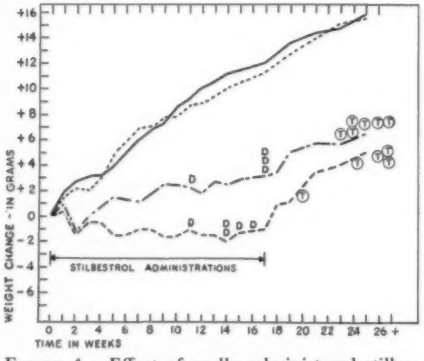

FIGURE 1.—Effect of orally administered stilbestrol in C3H male mice.

Solid line, 0.1 cc. of sesame oil twice a week.

Dotted line, untreated controls.

Dash-dot line, 0.125 mg. of stilbestrol twice a week.

Short-dash line, 0.375 mg. of stilbestrol twice a week.

Letters D and T represent death of mouse and mouse with mammary tumor, respectively.

Subcutaneously, 0.5 mg. of stilbestrol twice a week killed 7 of 10 mice in 2 to 5 weeks, and another mouse died 7 weeks after the first injection. With 0.25 mg., the mortality was 3 of 10 mice in 9 to 11 weeks, and 2 mice of 10 died in 10 weeks when 0.125 mg. was given twice a week. In the experiment reported previously (*1*), C3H males tolerated 0.25 mg. of stilbestrol in 0.25 cc. of sesame oil injected subcutaneously once a week for 24 weeks. The mortality with 0.125 mg. in the present series is attributed to the frequency of the administrations.

Retardation in weight increment or actual loss of weight were seen consistently with the doses of stilbestrol employed. The effect was directly proportional to the dose of the compound administered (fig. 1 and table 1), and was more marked when stilbestrol was injected subcutaneously than when it was given by mouth (tables 1 and 2).

The retardation in weight increment probably was a manifestation of disturbance in growth, since the mice were small in size as well as light in weight. Similar results, elicited with natural as well as synthetic estrogens, have been reported by Noble (*5*), Gaarenstroom and Levie (*6*), and Kochakian (*7*). Gaarenstroom and Levie also noted that in rats stilbestrol caused arrest of bone development par-

TABLE 1.—*Effect of oral administration of stilbestrol on body weight and food consumption in C3H male mice*

Stilbestrol[1] per dose	Number of mice	Average weight, according to week of administration									Total gain
			0	1	2	3	4	5	6	7	8	
Milligrams			*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
	5	Weight of mice	21.1	23.5	24.2	24.0	25.1	25.3	26.0	26.7	26.9	5.8
		Food per gram body wt./day	.15	.15	.14	.14	.14	.14	.13	.13	.13	
0.375	5	Weight of mice	21.3	23.6	23.1	22.6	22.8	22.6	22.7	22.2	22.1	.8
		Food per gram body wt./day	.16	.15	.15	.16	.14	.14	.14	.13	.14	
0.75	5	Weight of mice	20.9	22.4	22.4	21.6	21.7	21.8	21.4	21.2	21.1	.2
		Food per gram body wt./day	.15	.15	.14	.15	.14	.14	.14	.13	.13	

[1] The stilbestrol was dissolved in 0.1 cc. of sesame oil and administered twice a week.

ticularly at the epiphyseal junction and stated that unlike a similar effect with estrone, the arrested osseous development with stilbestrol was not counteracted by pituitary growth hormone.

Tables 1 and 2 demonstrate that the retardation in gain of weight or growth was not the result of decreased food consumption but was due to some other action of the compound. The intake of food per gram of body weight was the same in the mice that received stilbestrol as in the controls. With the discontinuation of stilbestrol administrations there was a definite increase in weight (fig. 1), but the mice remained lighter and smaller than the untreated controls throughout the period of observation.

In the previous report on the effect of stilbestrol in C3H mice (*1*), no morphologic changes were noted except in the mammary and the genital tissues. The highest dose used, however, was 5.0 mg. injected during 24 weeks, and only two animals died during the course of, or soon after, the termination of the administrations. The present series include higher dose ranges, and observations on the immediate effects of the compound are recorded.

The pathologic findings were the same whether the compound was administered subcutaneously or orally, and there were no differences with the various doses employed except that the changes were more marked with the higher doses. In the C3H male mice the chief effects of large doses of stilbestrol were (1) loss of weight, (2) atrophy of the viscera, particularly of the spleen, (3) decreased spermatogenesis in the testes, (4) development of slight scrotal herniation, and (5) development of mammary carcinoma.

The most striking lesion observed in animals dying early in the course of the experiments was generalized atrophy of

TABLE 2.—*Effect of subcutaneous injections of stilbestrol on body weight and food consumption in C3H male mice*

Stilbestrol[1] per dose	Number of mice	Average weight, according to week of administration										Total gain
			0	1	2	3	4	5	6	7	8	9	
Milligrams			*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
	10	Weight of mice	25.6	26.7	26.9	28.2	29.0	29.2	30.4	31.5	31.7	32.4	6.8
		Food per gram body wt./day	.12	.10	.11	.10	.09	.09	.08	.08	.08	.08	
0.125	10	Weight of mice	25.0	24.9	25.6	25.5	25.7	24.6	24.8	25.1	25.8	25.7	.7
		Food per gram body wt./day	.12	.12	.13	.12	.12	.11	.12	.12	.11	.11	
0.25	10	Weight of mice	25.0	25.1	24.6	25.0	24.5	24.9	25.1	25.6	24.7	24.7	−0.3
		Food per gram body wt./day	.10	.10	.11	.10	.11	.10	.10	.10	.10	.11	

[1] Stilbestrol was dissolved in 0.1 cc. of sesame oil and injected twice weekly.

the viscera, particularly of the spleen which was sometimes reduced to approximately one-half its normal size. Atrophy involved chiefly the pulp and to a lesser degree the splenic follicles. The testes were also slightly smaller than normal and histologically revealed moderate degrees of spermatogenic arrest. The interstitial cells however, exhibited only minimal hyperplasia and pigmentation. The fluid content was frequently diminished in the seminal vesicles, and squamous metaplasia in the coagulating glands was either absent or limited to the proximal portions of the ducts. No evidence of gross or microscopic lesions of the liver was observed.

In animals which survived 18 weeks or longer, the incidence of lesions was inconstant save for the occurrence of mammary adenocarcinoma. Slight hyperplasia of interstitial cells of the testes occurred usually in association with arrest of spermatogenesis but did not approach in degree similar lesions seen in mice of strains C or C57 Black which had received stilbestrol-cholesterol pellets (*8*). Scrotal hernias were infrequently seen, although in one instance strangulation of the testis in the hernial sac occurred. Squamous metaplasia of the coagulating glands, fibromuscular hyperplasia of the seminal vesicles, and myxomatous degeneration of the ejaculatory ducts were seen for the most part in those animals dying or killed within a few weeks after the cessation of treatment. These changes are apparently reversible, since in all the animals living 10 months or more the genital organs showed no abnormality.

In all animals which lived to the age of 10 months "brown degeneration" of the adrenals was noted but was not seen in animals which died during the period of treatment. The pituitary gland was examined in all instances, but in no case did it appear enlarged. The acini of the thyroid gland were frequently lined by cuboidal epithelium and filled with acidophilic colloid. No significance is attached to these findings in view of the structural variations noted in untreated animals of this strain.

In none of the C3H mice which received up to 14.75 mg. of stilbestrol in sesame oil, or in mice of strains C, C57 Black, or A into which stilbestrol-cholestrol pellets (*8*) containing up to 3 mg. of the compound were implanted subcutaneously, was there evidence of hepatic damage. Loeser (*9*) and others (*10*) described hepatic necrosis in rats that received stilbestrol in doses of 1 mg. thrice a week to once a day. Selye (*11*) reported marked icterus, necrosis, and leukocytic infiltration of the liver, as well as hemorrhagic pneumonia and gastric ulcers, in mice that were injected with 1 mg. daily or with 5 mg. once a week for a total dose of up to 15 mg. of stilbestrol. Although the doses were higher than employed in this study, Selye's description of the hepatic damage is similar to that seen in a spontaneous infectious disease of mice, known commonly "as liver disease."

The toxic and other effects of stilbestrol have been summarized recently by Von Haam and his coworkers (*10*, *12*). With large doses, it is apparently well-substantiated that stilbestrol causes a disturbance in the growth of mice and of rats (as manifested by the effect on weight increment and the epiphyseal junctures), hepatic focal necrosis in rats, and anemia and thrombocytopenic purpura in dogs. The effects are not unique for stilbestrol but occur with other estrogens of comparable potency. Stilbestrol, however, retains its potency by the oral route of administration, whereas natural estrogens do not. Increasing evidence is accumulating (*13*, *14*) that estrogens are detoxified by the liver and that in the presence of hepatic

damage the effect of the hormone is more pronounced. Zondek and Sulman (*15*) showed that the rat is unable to inactivate considerable amounts of stilbestrol, which would explain its effectiveness by the oral route and its eventual toxic activity with large doses.

The effects of stilbestrol and other estrogens in the mouse vary widely, both qualitatively and quantitatively, depending upon the strain of mouse employed. Bonser and Robson (*16*) and the observations at this laboratory, for example, have shown that the C57 Black mice develop extreme degrees of urinary retention and scrotal herniation, whereas these effects are much milder in mice of strain A or C3H. Also, whereas C57 Black mice develop pituitary adenomas (*17*) and strain A and C mice develop primary testicular tumors with estrogens, these lesions are not elicited in strain C3H mice.

Tumors

Table 3 presents the data on the induction of mammary carcinomas in male C3H mice that ingested stilbestrol for 15 to 17 weeks. Of the effective total of 22 animals, 18 developed tumors of the breast in an average period of 24 to 27 weeks following the administration of 4.25 to 14.25 mg. of the compound. There was no decrease in the latent period, as determined by the appearance of palpable tumors, with the higher doses. It is possible that, as Burns and Schenken (*18*) showed for estrone, 5.0 mg. administered during 15 weeks represents the maximum effective dose and length of time for the compound in its ability to evoke mammary carcinoma. The average latent periods in this series agree closely with the average of about 28 weeks observed in the previous experiment (*1*) in which the compound was given subcutaneously (5 mg. in the course of 24 weeks).

The appearance histologically of these mammary tumors is that of adenocarcinoma described previously (*1*).

No tumors of the lymphatic system were observed in any of these experimental groups. In an unrecorded experiment,[3] however, 30 C3H male mice lived for 6 months or longer after the subcutaneous implantation of crystals of estrone weighing 1.5 to 2.0 mg. Two of these mice, in 6 and 8 months after the administration of the hormone, respectively, developed gross enlargement of the spleen and lymph nodes with massive hyperplasia of small, round cells with darkly staining nuclei. There was complete obliteration of the normal pattern of both spleen and nodes but no evident invasion of the other organs. These findings are in agreement with those of Gardner, Kirschbaum, and Strong (*19*), who obtained lymphoid tumors in another subline of strain C3H mice.

[3] *Andervont, H. B., and Skimkin, M. B.: Unpublished data.*

Table 3.—*Induction of mammary carcinomas in C3H male mice following biweekly oral administration of stilbestrol dissolved in 0.1 cc. of sesame oil*

Stilbestrol per dose	Administrations	Stilbestrol total dose	Number of mice	Mammary tumors, in weeks after initial administration										Total tumors	Average time
				18	20	22	24	26	28	30	32	34	36		
Milligrams	*Number*	*Milligrams*		*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*Number*	*Weeks*
0.125	34	4.25	7				3	1	1	1				6	25.7
0.375	15	8.625	5	1		1					1			3	23.7
0.2	15														
0.375	34	12.75	5		1		1	1			1		1	5	27.4
0.75	15	14.25	5			2				2				4	26.0
0.2	15														

SUMMARY

In strain C3H mice the chief effects of large doses of orally or subcutaneously administered stilbestrol were (1) loss of weight that was not due to decreased food consumption but probably represented retardation in growth, (2) atrophy of the viscera, particularly of the spleen, (3) decreased spermatogenesis in the testes, (4) development of slight scrotal herniation, and (5) development of mammary adenocarcinoma. Hepatic lesions were not observed even with lethal doses of the compound.

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The Lack of Carcinogenic Potency of Desoxycorticosterone in Mice

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The property of estrogens of inducing or accelerating the appearance of mammary carcinomas and other tumors in mice of susceptible strains has indicated the desirability of investigating other hormones in their relation to the development of neoplasia. The experiment reported herein was performed with desoxycorticosterone acetate. This hormone is of particular interest since—

in addition to its activity as an essential adrenal cortical hormone, it will stimulate mammary development somewhat after the manner of the estrogens, it has progesterone-like activities and is androgenic (*1*).

EXPERIMENTAL PROCEDURE

For subcutaneous injections, strain C3H mice of a subline maintained at the National Cancer Institute were used (*2*). Practically 100 percent of the virgin females develop spontaneous mammary tumors at an average age of 11 months. Breeding or injections of estrogens reduce the average age of tumor development to 7 or 8 months. The males develop mammary tumors following treatment with estrogens (*3*). The mice are susceptible to the induction of subcutaneous sarcomas with carcinogenic hydrocarbons (*4*).

The mice were maintained on a standard diet containing 1.4 percent of sodium chloride [1] and had access to an unlimited supply of water. They were 2 to 3 months old at the beginning of the experiment.

Crystalline desoxycorticosterone acetate [2] was dissolved in sesame oil, 10 mg. per cubic centimeter. Subcutaneous injections of 0.1 or 0.05 cc. were given twice a week for the first 2 months, and once a week for the following 6 months. All mice received 49 injections in 35 consecutive weeks, for the following total doses: Group A, 10 C3H males, 33 mg.; group A1, 10 C3H males, 21.5 mg.; group C, 12 C3H females, 33 mg. Three groups served as controls: Group B, 10 C3H males, and group D, 9 C3H females, which received 3.3 cc. of sesame oil subcutaneously during 35 weeks; and group E, 10 untreated C3H females. The mice were weighed and examined for the presence of subcutaneous tumors once a week.

For intravenous injections, strain A male mice 2 to 3 months of age were used. These mice are very susceptible to the induction of primary pulmonary tumors with carcinogenic hydrocarbons and other agents (*5*). Desoxycorticosterone acetate was dispersed in a colloidal solution of cholesterol in distilled water to which was added 0.03 percent of dioctyl ester of sodium sulfosuccinate, to equal 1 mg. of the hormone per cubic centimeter of the vehicle.[3]

Seventeen strain A males received in the lateral tail vein 0.2 cc. of the dispersion five times at weekly intervals, for a total dose of 1 mg. Twenty untreated A males were maintained as controls. The mice were killed 20 weeks after injection, and

[1] *Devised by Dr. H. P. Morris. The diet consisted of milk powder, 22.75 percent; graham flour, 61.52 percent; brewers' yeast, 4 percent; iron citrate, 0.13 percent; cod-liver oil, 2 percent; corn oil, 8.2 percent; and sodium chloride, 1.4 percent.*

[2] *We are indebted to Dr. Erwin Schwenk, of the Schering Corporation, and to Dr. B. L. Josephy, of Roche Organon, for the supply of the compound.*

[3] *Prepared by Dr. Egon Lorenz according to his method, described elsewhere* (6).

the lungs were examined for the presence and the number of primary pulmonary tumors (*5*).

RESULTS

All of the C3H mice tolerated the subcutaneous injections of desoxycorticosterone and were alive 9 months after the beginning of the experiment. Both the males and the females that received the hormone maintained a higher average weight than the comparable controls receiving sesame oil (fig. 1). This effect on weight was perhaps a manifestation of the water retention that has been demonstrated to occur with desoxycorticosterone (*7*).

Of the 32 mice that received desoxycorticosterone subcutaneously, 21 were living at 1 year after the initiation of the injections. None developed subcutaneous sarcomas at the site of injection. At 14 months 10 male mice were living without tumors and were killed to terminate the experiment. Of these 10 mice, 16 to 17 months of age, 3 had hepatomas. The incidence of hepatomas is the same as that reported to occur spontaneously (*2*).

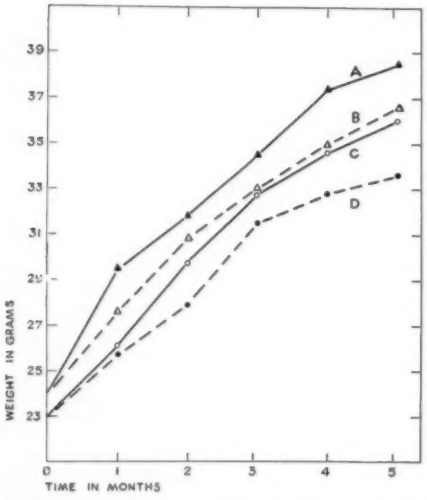

FIGURE 1.—Effect of desoxycorticosterone acetate on the weight increment of C3H mice.

A, 10 C3H males injected subcutaneously with 1.0 mg. of desoxycorticosterone weekly or twice a week.
B, 10 C3H males injected subcutaneously with sesame oil.
C, 12 C3H females injected subcutaneously with 1.0 mg. of desoxycorticosterone acetate weekly or twice a week.
D, 9 C3H females injected subcutaneously with sesame oil.

The appearance of mammary tumors in the females is recorded in table 1. It is evident that desoxycorticosterone did not accelerate the appearance of mammary carcinoma in strain C3H female mice. No mammary tumors appeared in the male mice.

Of the 17 strain A male mice that were killed 20 weeks after the intravenous injection of 1 mg. of desoxycorticosterone, 4 had single pulmonary tumors. Of the 20 control animals, 6 developed primary pulmonary tumors. Thus, the hormone did not increase the incidence of this type of neoplasm in mice of a susceptible strain.

No gross or microscopic abnormalities were observed upon examination of the adrenal, pituitary or thyroid glands, gonads, liver, or spleen. The animals were not killed during the period of treatment, however, and reversible morphologic alterations may have disappeared. The mammary tumors of the female and the hepatomas of the male C3H mice as well as the pulmonary tumors of strain A mice were essentially similar to those occurring spontaneously in these animals (*3, 5, 8*).

SUMMARY

Weekly or biweekly subcutaneous injections of desoxycorticosterone acetate in sesame oil (up to 33 mg. in 35 weeks) did not accelerate the appearance of mammary carcinomas in strain C3H female mice and did not induce subcutaneous

TABLE 1.—*Occurrence of mammary carcinomas in strain C3H virgin females injected with desoxycorticosterone acetate in sesame oil*

Group	Injection	Number of mice	Mammary tumors, according to age of mice (in months)								Total tumors	Mean tumor age
			8	9	10	11	12	13	14	15		
											Number	*Months*
C	Desoxycorticosterone acetate, 33 mg	12	.....	3	1	1	3	2	.....	1	11	11.3
D	Sesame oil, 3.3 cc	9	1	2	1	2	1	1	1	.....	9	10.7
E	Untreated	10	1	1	1	2	3	1	1	.....	10	11.1

sarcomas or other tumors in female or male mice of this strain during 14 months of observation. Intravenous injection of 1 mg. of desoxycorticosterone acetate into strain A mice did not induce primary pulmonary tumors.

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Induction of Testicular Tumors and Other Effects of Stilbestrol-Cholesterol Pellets in Strain C Mice

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In this communication is reported the reaction of mice of strain C and strain C mice foster nursed by C3H females to stilbestrol. Particular reference is made to the development of a method of administering the estrogen as a single implantation in the form of stilbestrol-cholesterol pellets, and to the induction of primary testicular tumors of the interstitial-cell type. During the preceding year (1940), two reports, one by Bonser and Robson (*1*) and the other by Hooker, Gardner, and Pfeiffer (*2*), described the induction of primary interstitial-cell tumors of the testis in strain A mice following massive treatment with estrogens. Two of the reported tumors metastasized to the regional lymph nodes.

Strain C mice are an albino stock inbred by Dr. E. C. MacDowell from animals originally received from Dr. H. G. Bagg. The mice used in the present investigation are descendants of an F_{35} litter obtained from Snell (*3*), of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The colony, continued by brother-to-sister matings, is now in the F_{48} to F_{50} generations.

Strain C mice that are maintained at the National Cancer Institute develop a high incidence of spontaneous lymphoid tumors after the age of 16 months; the incidence rises gradually to about 50 percent at the age of 24 to 27 months. Pulmonary tumors begin to appear when the animals are 10 to 11 months of age, and the incidence rises to about 30 percent when they are 18 to 24 months old. The breeding females have an incidence of less than 2 percent of mammary carcinoma; but when they are foster nursed by C3H females, the incidence rises to over 60 percent (*4*). Spontaneous tumors involving the salivary glands occur in the stock. Spontaneous or induced tumors of the testis have not been described or noted in this strain.

EXPERIMENTAL PROCEDURE

The usual method of administration of estrogens and other hormones in this type of experiment is the long-continued subcutaneous injection of oily solutions. The procedure is tedious to the investigator and deleterious to the animals. Attempts to use single implantations of solid crystals of estrone (*5*) or stilbestrol proved unsatisfactory in our hands, since the small size of the crystals made it difficult to handle and to weigh them accurately, and with crystals weighing 1.5 mg. or more there was prohibitive mortality among the mice.

In order to reduce the concentration of the estrogen and to develop a more adequate method of administering this and similar compounds in experiments on carcinogenesis, stilbestrol-cholesterol pellets were prepared according to the method used by Shear (*6*) in making pellets of carcinogenic hydrocarbons. Stilbestrol [1]

[1] *4,4'-dihydroxy-α,β-diethylstilbene, furnished by Merck & Co.*

and cholesterol were melted in an oil bath at a temperature of 160° to 170° C. The solution was drawn up into an oiled glass tube with a 2-mm. bore, with a well-fitting wire as a plunger. As soon as the material solidified, it was extruded from the glass tube. The resultant solid rod of stilbestrol in cholesterol was divided into pellets by means of a razor blade. The pellets were then washed in ether and in alcohol to remove the liquid petrolatum on the external surface and to smooth the rough edges.

Pellets containing 5, 10, 25, and 50 percent of stilbestrol, and weighing 4 to 6 mg. were thus prepared, as well as pellets of solid stilbestrol.

The pellets were introduced through a trocar into the subcutaneous tissue of the right axilla of strain C mice. The animals were about 6 weeks old at the beginning of the experiment in May 1940. Some groups, however, included animals that were up to 3 months of age. Approximately equal numbers of male and female mice were used in each group, as given in table 1. The table includes a group of 20 females and 13 males that were foster nursed by strain C3H females. Discrepancies between the total numbers of mice and the numbers given in subsequent tables are owing to the fact that some of the animals were killed before the appearance of tumors.

TABLE 1.—*Mortality in strain C mice following the subcutaneous implantation of 4- to 6-mg. pellets of stilbestrol in cholesterol*

[Results up to 6 months]

Concentration of stilbestrol in cholesterol (percent)	Males			Females		
	Number of mice	Deaths		Number of mice	Deaths	
		Number	Time		Number	Time
			Weeks			*Weeks*
5	10	0	------	10	0	------
10	45	0	------	10	0	------
25	24	1	20	26	1	9
50	14	3	14-20	24	14	2-13
100	14	14	3-6	14	14	2-5

The mice were maintained in wooden boxes on a diet of Purina dog-chow pellets and had access to an unlimited supply of water. The males and females were kept separate. The animals were examined weekly, and groups of 10 males and 10 females injected with each concentration of stilbestrol, as well as groups of untreated controls, were weighed once a week for the first 14 weeks of the experiment. The investigation was terminated 11 months following the administration of the stilbestrol pellets, when the mice were 13 to 14 months old.

RESULTS

The use of stilbestrol-cholesterol pellets has proved gratifying. The pellets remain in the subcutaneous tissue of the mice for at least 11 months without gross reduction in size other than slight erosion at the edges. Solid stilbestrol pellets, however, became definitely reduced in size in the mice that survived 3 or more weeks after implantation. Forbes (7) implanted solid pellets of stilbestrol in the subcutaneous tissue of rats and found that there was 90-percent absorption in 51 days. His pellets had been made by compression and weighed 6 to 10 mg. The slow absorption of stilbestrol-cholesterol pellets in mice is attributed to the presence of the cholesterol.[2]

Most of the stilbestrol-cholesterol pellets became thinly encapsulated by the termination of the experiment, but the fibrous reaction in all cases was mild.

[2] *Similarly, pellets of 25 percent of testosterone in cholesterol have been observed grossly unchanged in the subcutaneous tissue of the mouse for at least 4 months, which suggests that the combination of hormones with cholesterol may be proficient in reducing the rate of absorption of hormones implanted in solid form. Experiments on the absorption rate of such pellets are now in progress.*

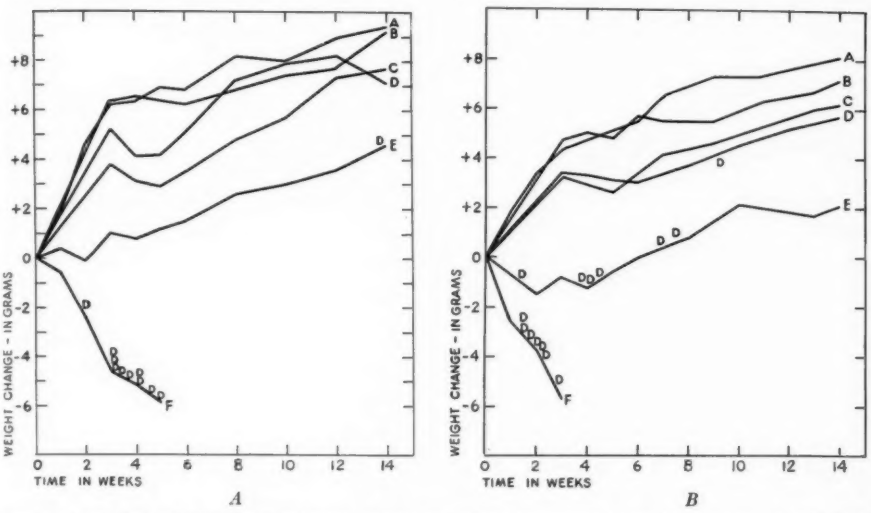


FIGURE 1.—Effect of stilbestrol-cholesterol pellets on weight increment in strain C mice. *A*, males; *B*, females.

A, untreated controls.
B, 5-percent stilbestrol pellet.
C, 10-percent stilbestrol pellet.
D, 25-percent stilbestrol pellet.
E, 50-percent stilbestrol pellet.
F, solid stilbestrol pellet.
Letter D on weight lines represents death of mouse.

EFFECT ON WEIGHT

The first effect of the implantation of the pellets to be noted was a marked retardation in weight increment that was roughly proportional to the concentration of stilbestrol in the pellet. The influence of stilbestrol on the weight of strain C mice is shown in figure 1.

It has been shown in strain C3H mice that this retardation in gain in weight is not caused by loss of appetite, since the mice that lose or do not gain weight ingest the same amount of food per gram of body weight as do the normal controls (*8*).

MORTALITY

Strain C mice tolerate pellets containing up to 25 percent of stilbestrol without significant mortality, as shown in table 1. The females appear to be somewhat more susceptible to the compound. The average weight of the females at the beginning of the experiment, however, was 19.1 gm. as compared with 20.4 gm. for the males. When 50- and 100-percent stilbestrol pellets were given, there was a high mortality among the animals.

The lethal effect of stilbestrol pellets varies significantly in different strains of mice, probably because of the obvious differences in the effect of the compound on these strains. Strains C and C3H mice tolerate 25-percent pellets with a mortality of less than 10 percent; they lose weight, and there are atrophy of the spleen and other organs and minor degrees of urinary retention. Strain C57 Black mice have a high mortality even with 10-percent pellets of stilbestrol, because of the early development of extreme urinary retention. Among the male survivors there is a high incidence of large scrotal hernias, some of which

become incarcerated. Strain A mice, on the other hand, have a relatively late mortality of about 20 percent following the administration of 25-percent stilbestrol pellets because of urinary retention which produces paradoxical incontinence and extensive ulceration of the perineum because of urinary dribbling.

PATHOLOGIC OBSERVATIONS

The pathologic findings were the same qualitatively with all concentrations of stilbestrol, but the changes were more marked with the higher concentrations.

Female Genitourinary System

The females that died during the first month after the implantation of the 50- and 100-percent stilbestrol pellets showed acute endometritis and hyperplasia of the endometrium.

Frank pyometra, however, occurred in only 3 of 44 females that died or were killed during the first 6 months of observation.

Two months or later after the implantation of the pellets there was a consistent cystic hyperplasia of the endometrium of extreme degree and frequent hyalinization of the endometrial stroma. Hyperplasia and hyperkeratosis of the vaginal mucosa occurred in the majority of the animals, but no invasive lesions were encountered during the 11 months of the experiment. The ovaries were uniformly active, and two mice developed small follicular cysts. No qualitative differences in the appearance of the ovaries were encountered when strain C females were compared with C mice that were foster nursed by C3H females. This indicates that the morphologic differences between ovaries of a strain susceptible to mammary carcinoma (dba) and one resistant to this tumor (C57 Black) as described by Taylor and Waltman (*9*) are probably not etiologically related to the degree of disposition to cancer of the breast.

A surprisingly mild effect upon the urinary system was noted, considering the marked reaction in mice of strain C57 Black. A few of the females developed urinary retention, but there was only one case of cystitis and hydronephrosis, 5 months after the implantation of a 25-percent pellet.

Male Genitourinary System

The effects of stilbestrol on the genital tissues of the male during the first month consisted of degenerative changes in the seminiferous epithelium with partial arrest of spermatogenesis, and fibromuscular hyperplasia of the seminal vesicles which decreased in size because of the absence of secretion, and varying degrees of squamous metaplasia of the prostatic gland complex, particularly the coagulating glands.

There was no appreciable proliferation of interstitial cells in the testes during the first month. At 2 months the cells were sufficiently pigmented to give the organ a dark-brown cast on gross inspection. The proliferation of the interstitial cells at this stage was diffuse but resulted in no evident increase in the size of the organ because of the concomitant shrinkage of the tubules.

About 10 percent of the males developed scrotal hernias following the administration of 10- to 50-percent stilbestrol-cholesterol pellets. The hernias were usually on the left side and contained the terminal portion of the cecum. In only one case, in which death was due to perforation of a loop of intestine in the hernial sac, was the herniation of large size. In the mice that developed testicular tumors, however, the hernias were larger and in one instance the sac contained the bladder. Even in these mice the extent of the herniation did not

approach that seen in C57 Black males following the same treatment.

Marked urinary retention and secondary obstructive lesions were not observed in strain C males.

Testicular Tumors

Three to four months after the implantation of the stilbestrol pellets, the male C mice developed atrophy of the seminiferous tubules, almost complete lack of spermatogenesis, and diffuse hyperplasia of the interstitial cells. Plate 1, *A*, presents the appearance of a normal testis of a strain C mouse, for comparison with the effects shown in plate 1, *B*, 3 months after the administration of a 25-percent stilbestrol pellet. The interstitial cells appeared as cords or small masses of large mononuclear cells frequently arranged about a small blood vessel and with a characteristically vacuolated or pigmented cytoplasm. The cells were appreciably larger than those in the normal testis, but mitotic figures were rare. In routine haematoxylin-eosin preparations or in unstained frozen sections, the pigment formed diffuse, yellowish-brown deposits in the cytoplasm and stained black in osmicated preparations and reddish orange with sudan IV in both frozen and paraffin sections. The pigment was doubly refractile when examined with the polarizing microscope.

Four to five months after the injection of pellets containing 10 percent of stilbestrol or more, the testes showed nodular hyperplasia of interstitial cells which could be seen grossly through the white scrotums of the mice, and appeared as white spots on the yellow-brown testes. The majority of these hyperplastic areas were directly subcapsular (fig. 2), but some also appeared in the center of the organ (pl. 2, *A*). While the cytoplasm in these nodular proliferations was frequently vacuolated, the yellow-brown pigment was notably less than that observed in the more diffuse interstitial hyperplastic areas, and not infrequently interstitial cells completely devoid of pigment and with smooth, palely acidophilic cytoplasm were observed.

The hyperplasia of the interstitial cells progressed to practically complete replacement of the seminiferous tubules by 6 months after the implantation of stilbestrol, as shown in plate 2, *B*.

By this time practically all the mice had some degree of hyperplasia of the interstitial cells, and the designation of the time of appearance of frank tumor was difficult. Considering enlargement of the testis to at least twice normal size and the presence of large nodularities as the minimal criteria for tumor, 13 testicular tumors appeared by 11 months after the implantation of the hormone pellets. Table 2 reveals that the incidence of the lesions was roughly correlated with the concentration of the stilbestrol in the pellet. Three of thirty mice receiving 10-percent pellets and five of eight mice that received 50-percent pellets developed testicular tumors.

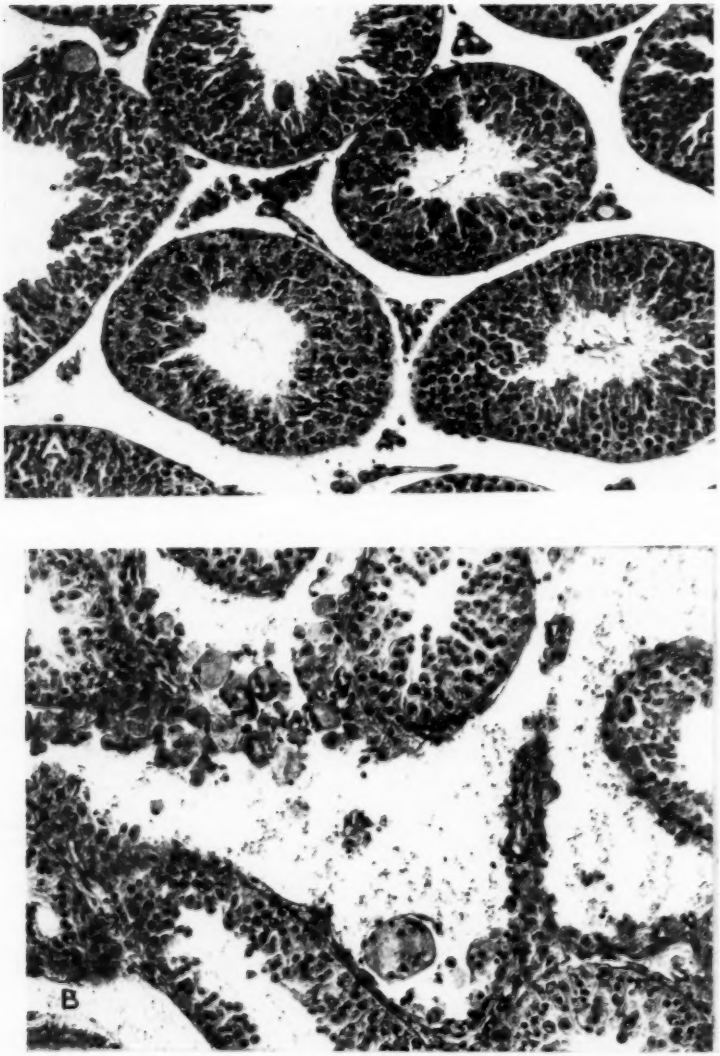
Grossly the testicular tumors appeared as large, frequently pear-shaped masses of characteristic brownish-yellow color (pl. 3, *A*). They were not adherent to the

Table 2.—*Induction of primary testicular tumors in male mice of strain C following the subcutaneous implantation of stilbestrol-cholesterol pellets weighing 4 to 6 mg.*

Concentration of stilbestrol in cholesterol (percent)	Effective total of mice	Tumors, according to months after pellet implantation						Total tumors
		6	7	8	9	10	11	
	Number	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*Number*
5	10	...	...	...	...	...	1	1
10	30	...	[1] 1	1	1	...	...	3
25	14	1	2	...	...	[1] 1	...	4
50	8	1	2	...	...	[1] 1	1	5

[1] Metastases present.

PLATE 1



A, Testis of normal strain C mouse 11 months old. Note distribution of interstitial cells. Haematoxylin and eosin. ×175; *B*, hyperplasia and pigmentation of interstitial cells in testis of strain C mouse 3 months after implantation of 25-percent stilbestrol pellet. Haematoxylin and eosin. ×175.

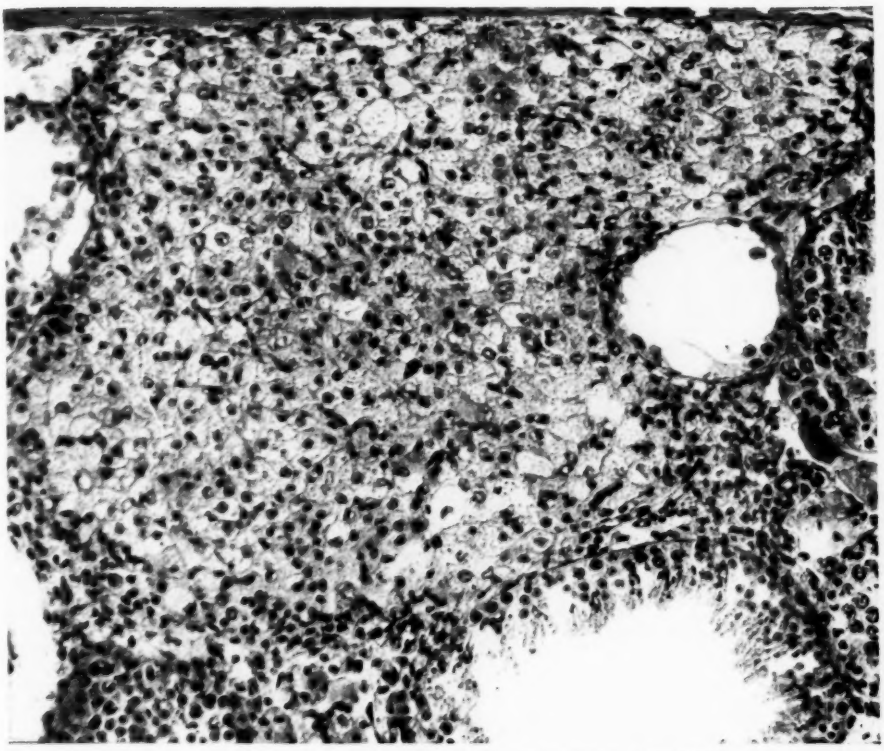


FIGURE 2.—Testis of strain C mouse 4 months following the subcutaneous implantation of a 25-percent stilbestrol-cholesterol pellet. Note the nodular subcapsular hyperplasia of interstitial cells. Haematoxylin and eosin. × 210.

surrounding tissues and could be freed from the scrotums by gentle traction. The capsules were extremely vascular, and the vessels frequently were greatly distended. In three instances the tumors had penetrated the capsule and invaded the genital omentum. The cut surfaces were soft and usually bulged above the capsule. The smaller tumors were white or very pale yellow and contrasted sharply with the surrounding dark-brown tissue. As the tumors grew larger, hemorrhagic and dark-brown necrotic foci became common. In one instance in which a large scrotal hernia was associated, the tumor was strangulated and apparently completely necrotic although a metastatic focus was found in the left suprarenal area. The epididymis was intact in all cases.

Histologically the tumors were composed of solid sheets or cords of large, polygonal, or slightly elongated cells supported by a sparse stroma. The tumor cells were almost invariably mononuclear, the nuclei being large, round, and moderately vesicular, and occasionally containing round acidophilic inclusion bodies. Mitoses were remarkably infrequent. The cytoplasm was pale and frequently smooth, although sudanophilic fat globules were not uncommon. Significantly smaller amounts of pigment were observed in the

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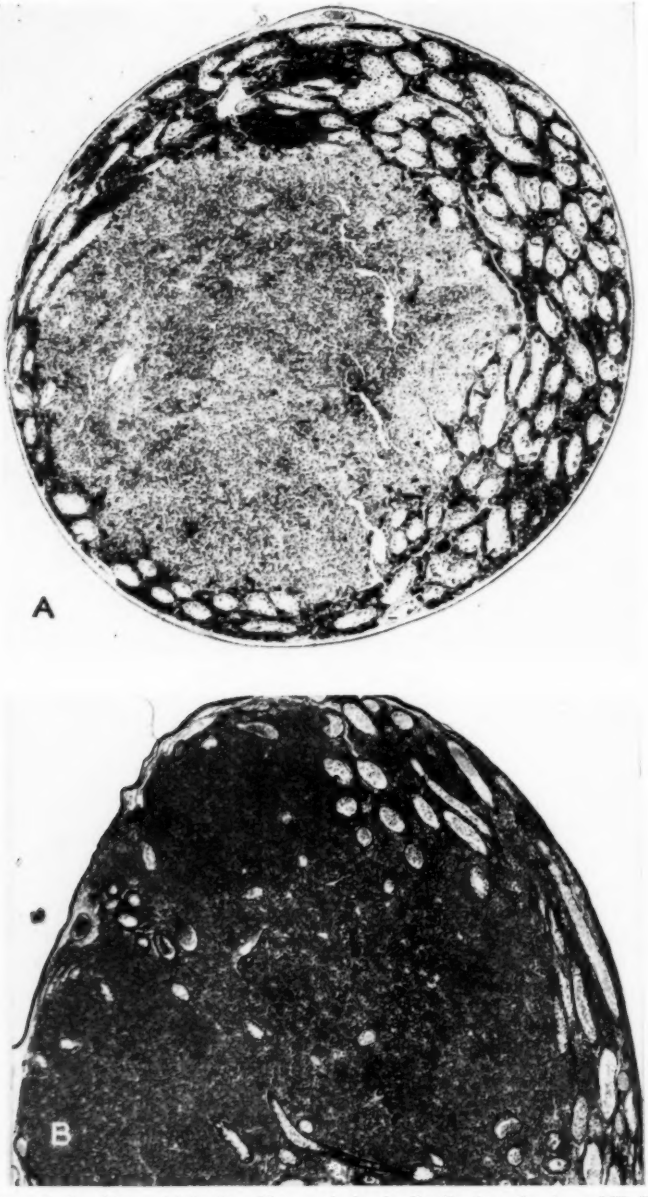
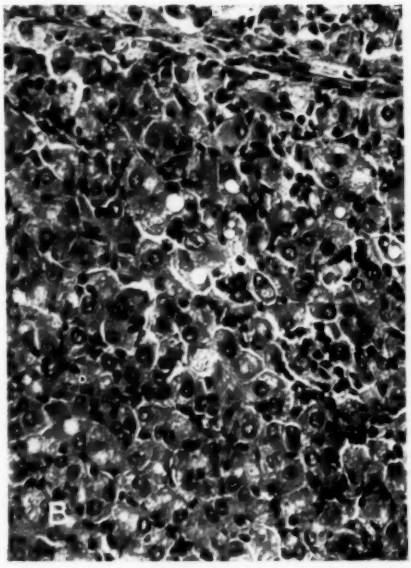
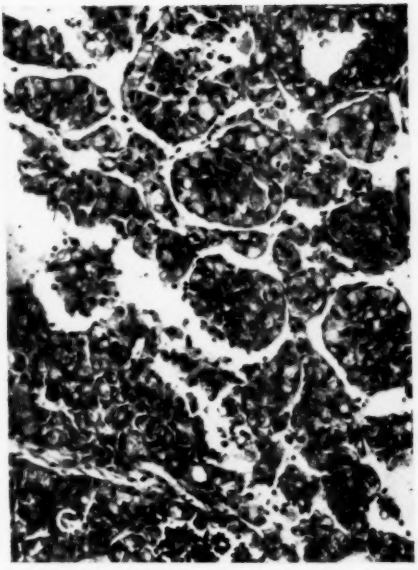
PLATE 2



A, Nodular hyperplasia of interstitial cells. Note variation in distribution of sudanophilic lipoid. Frozen section. Sudan IV. ×20; *B*, massive hyperplasia of interstitial cells with almost complete disappearance of tubules. Haematoxylin and eosin. ×20.

PLATE 3





A, Massive tumor of left testis with symmetrical enlargement of right testis. Seminal vesicles are small; *B*, interstitial-cell tumor growing in solid sheets. Note variation in cytoplasmic vacuoles. Haematoxylin and eosin. ×250; *C*, interstitial-cell tumor composed of cords covered by endothelium. Haematoxylin and eosin. ×130.

tumor cells than in the early stages of proliferation. Cytoplasmic crystals were never noted.

The topography of the tumor cells varied, but all types might be found in the same tumor. The most common arrangement was in the form of broad solid sheets in which minimal amounts of collagen and reticulum could be demonstrated (pl. 3, *B*). Not infrequently the tumor cells formed large cuffs about thin-walled blood vessels, but here also the sparse supporting tissue was for the most part immediately perivascular. Less frequently the cells formed solid round cords which were covered by thin flattened cells resembling endothelial cells and separated from each other by sinusoidal vascular channels (pl. 3, *C*). In three instances small foci of myeloid cells were observed, and in two of these similar myeloid elements were observed in the metastatic deposits. Necrotic and hemorrhagic areas were frequent in the larger growths, and in the strangulated tumor described above there was almost complete coagulation necrosis of all sections examined.

The capsular vessels were usually large and frequently varicose, but careful search failed to disclose vascular invasion or tumor emboli.

Two of the thirteen mice had bilateral testicular tumors. In the remaining 11 mice the other testis showed varying degrees of interstitial-cell hyperplasia.

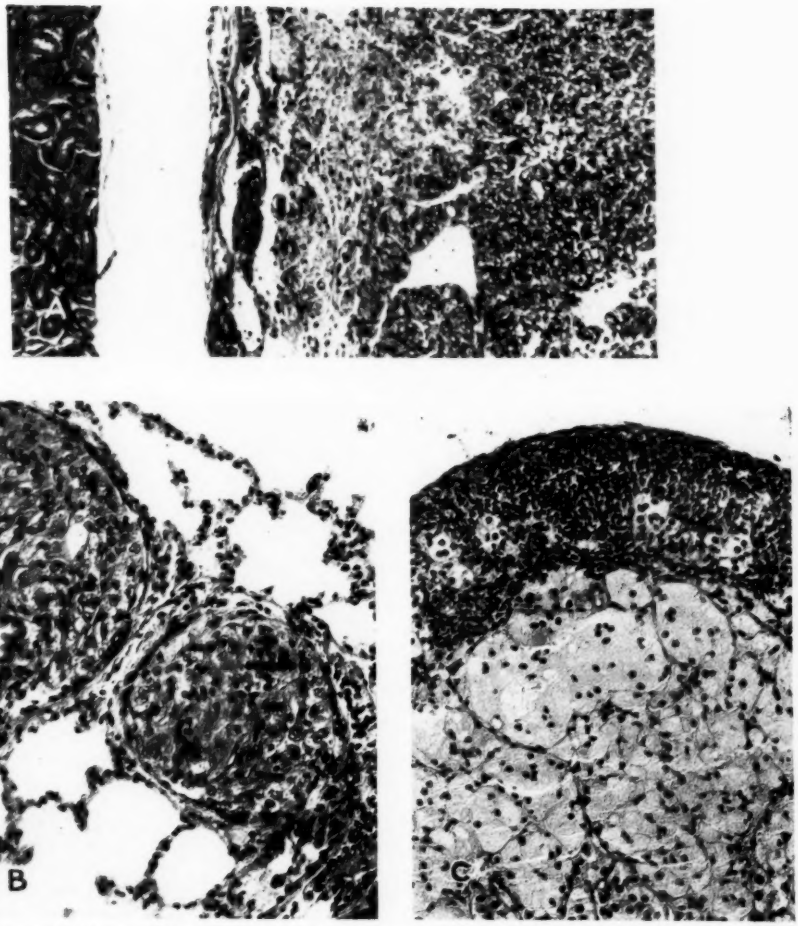
Of the 13 mice with testicular tumors, 3 had metastases to the region above the left kidney and posterior to the left adrenal. In 1 case the testicular tumor was on the right side, and in another both testes were replaced by tumor, but the metastases were limited to the left suprarenal region. The metastases were solid brownish-white masses, one of which was slightly hemorrhagic. There was no invasion of the adrenal or the kidney, and no structure resembling lymph node was present in the masses. The cells of the metastases reproduced the original tumor of the testis (pl. 4, *A*). In 1 case in addition to the suprarenal metastases, small embolic deposits were found in the peribronchial vessels of the lung (pl. 4, *B*). No metastases were found in the lumbar lymph nodes.

During the first 3 to 4 months there was uniform reduction in size of the seminal vesicles, but in the later stages in a considerable number of mice these organs were enlarged. Thus, of 15 mice killed 11 months after the implantation of 25- and 50-percent pellets, 5 had definite enlargement of the seminal vesicles. Whether this effect was a manifestation of the hormonal secretion of the interstitial cells is not clear, since the degree of hyperplasia was not correlated with the effect on the seminal vesicles. Thus, 2 mice with moderate, diffuse hyperplasia of the interstitial cells 11 months after the injection of the stilbestrol pellet had enlarged seminal vesicles, whereas 3 mice with testicular tumors had definitely small seminal vesicles.

The presence of testicular tumor did not preclude the development of mammary carcinoma in strain C males that were foster nursed by C3H females (*10*). Two of thirteen mice developed both mammary carcinoma and testicular tumor. The observations also indicate that foster nursing exerts no influence on the occurrence of primary induced interstitial-cell tumors of the testis in strain C mice.

As stated previously, there are two papers (*1*, *2*) in the literature concerning the induction of primary testicular tumors in strain A mice following long-continued treatment with large doses of estrogens. At the National Cancer Institute, 2 testicular tumors have appeared in 20 strain A (JAX) mice 7 months after the implanta-

PLATE 4



A, Metastasis of interstitial-cell tumor to region above left kidney. Haematoxylin and eosin. ×105; *B*, intravascular pulmonary metastasis of interstitial-cell tumor. Haematoxylin and eosin. ×200; *C*, brown degeneration and hyperplasia of zona glomerulosa of adrenal in male strain C mouse 11 months after implantation of 25-percent stilbestrol pellet. Haematoxylin and eosin. ×335.

tion of stilbestrol-cholesterol pellets. These tumors, as well as those elicited in strain C males, are of a pure interstitial-cell type. With the possible exception of 1 of the 28 primary tumors of the testis in 9,500 male mice reported by Slye, Holmes, and Wells (*11*), there are no descriptions in the literature of similar tumors occurring spontaneously in mice. Pure interstitial-cell tumors do occur spontaneously, however, in dogs and in horses; Peyron, Blanchard, and Salomon (*12*), for example found 4 such tumors in dogs and 2 in horses. The tumor occurs rarely in man. Hunt and Budd (*13*), in 1939, stated that there were 17 true or suspected human interstitial-cell tumors in the literature. In children they are often associated with manifestations of precocious puberty.

Adrenal, Pituitary, and Thyroid Glands

Four months and later after administration of stilbestrol, there was definite pigmentation of the cortico-medullary junction and occasionally of the cortex of the adrenal in practically all the animals. This pigment corresponded to that described by Cramer and Horning (*14*) as "brown degeneration" of the adrenal. The pigment was similar to that occurring in the testes but was not doubly refractile.

The incidence and the degree of this adrenal finding was identical in the male and female mice, and in the strain C mice as compared with those that had been foster nursed by strain C3H females. Since 70 percent of the foster-nursed females and 5 of 13 foster-nursed males developed mammary carcinomas whereas none of the normal males and 1 of 34 females developed tumor of the breast, this is additional evidence that the brown degeneration of the adrenal has no causal relationship to the development of mammary carcinoma.

Hyperplasia of the zona glomerulosa was a consistent finding. It was usually focal, but in one instance was diffuse and of such degree that the cortex was almost entirely composed of cells of the zona glomerulosa (pl. 4, *C*).

The pituitary gland remained of normal size in all mice of this experiment. The thyroid appeared normal grossly and histologically in all mice.

Spleen and Other Viscera

The loss of weight that occurred with large doses of stilbestrol (fig. 1) was accompanied in all cases by a generalized atrophy of the visceral organs. This atrophy was most marked in the splenic pulp. During the first 2 months the spleen was uniformly small, measuring in many instances 10 by 3 by 0.5 mm. (*8*).

During later stages, the surviving mice had spleens that were of normal size and frequently showed myeloid hyperplasia. Whether this finding was associated with the development of lymphoid tumors is not clear.

No hepatic lesions were encountered in the animals.

Lymphoid Tumors

Of 61 male and female strain C mice, comprising 4 separate groups and surviving 5 to 11 months after the implantation of stilbestrol pellets, 7 developed tumors of the lymphatic system (table 3). Of the 7, 4 had generalized, marked enlargement of the lymph nodes, involving the inguinal, axillary, abdominal, and mediastinal nodes, tremendous enlargement of the spleen, and moderate enlargement of the liver. The architectural pattern of the lymph nodes was obliterated by masses of small round cells, with single large round nuclei and scanty cytoplasm devoid of granules. Mitotic figures were numerous. Similar cells replaced the

splenic follicles and were found in large numbers in the pulp. The hepatic cell cords were narrowed in many areas by accumulations of these cells within the sinusoids, and in 1 animal similar infiltration was observed in the kidneys, ovaries, and lungs, as well as in large numbers in the visceral vessels. In 3 mice the mediastinal nodes alone were involved, and microscopically the lesions were similar to those described above. These mice died suddenly, possibly from tracheal compression.

TABLE 3.—*Appearance of lymphoid tumors in mice of strain C following the subcutaneous implantation of stilbestrol-cholesterol pellets weighing 4 to 6 mg., at the age of 6 weeks to 3 months*

Concentration of stilbestrol in cholesterol (percent)	Sex	Effective total of mice	Mice with tumors, according to age of mice (in months)							Total mice with tumors
			8	9	10	11	12	13	14	
		No.								*No.*
10	M	30		[1] L	L					2
25	M	16	[2] M							1
50	M	4				L				1
25	F	11			L		M		M	3
Total		61								7
Normal mice without tumors, according to age at death		143	3	8	16	33	26	20	37	0

[1] L, generalized lymph node and splenic enlargement.
[2] M, mediastinal lymph node enlargement only.

Strain C mice develop lymphoid tumors of this type spontaneously. Of 259 mice in the colony, no lymphomatoses have appeared in 143 mice that died or were killed when 8 to 16 months of age. When the mice are more than 16 months of age, lymphoid tumors begin to appear, the majority involving lymph nodes throughout the body, the spleen, and the liver; and the incidence rises to about 50 percent when the animals reach an age of more than 24 months (15 cases in 28 mice).

The mice injected with stilbestrol pellets in this experiment were not more than 14 months old when the experiment was terminated. The incidence of about 10 percent of lymphoid tumors at an average age of about 10 months permits the conclusion that the appearance of this neoplasm was accelerated by the experimental procedure (*15*).

MAMMARY CARCINOMAS

Only one 11-month-old female that received a 10-percent stilbestrol pellet 8 months previously developed mammary carcinoma. Of the remaining effective total of 34 females and 20 males, none developed this type of neoplasm up to 11 months of observation, when the mice were about 13 months old. Of 178 normal breeding females of the colony, 2 developed spontaneous mammary carcinoma at 24 and at 29 months of age.[3]

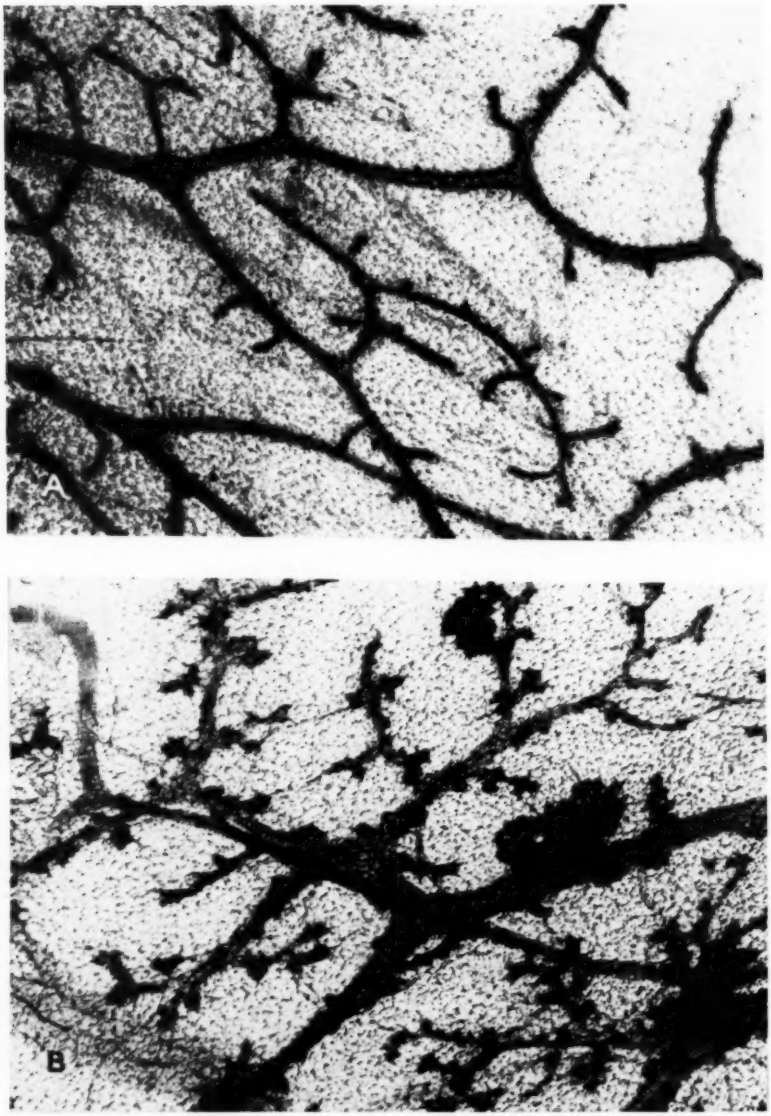
The incidence of mammary carcinoma was markedly increased when mice of strain C were foster nursed by C3H females (high-mammary-cancer strain). Of the effective total of 20 females, 14 developed mammary tumors at an average age of 8.4 months; and of the 13 strain C males fostered by C3H females, 5 developed tumors at an average age of 10.4 months. The significance of these results has been discussed previously (*16*). It is clear that the estrogens merely prepare the suitable substratum for the action of another factor, probably derived from the milk of the mother or foster mother.[4]

Comparison of mice of strain C with strain C mice that had been foster nursed by strain C3H females treated with stil-

[3] *Two females that received stilbestrol developed tumors involving the salivary glands, at 6 and at 10 months after the implantation of the pellets. They are presumed at present to be spontaneous in origin, as 2 similar tumors occurred in 259 untreated mice of the colony at 9 and at 19 months of age.*

[4] *That the stilbestrol-cholesterol pellets are satisfactory in inducing mammary tumors in male mice of strains susceptible to mammary tumors (i. e., strains that possess the milk factor) is indicated by the results in normal C3H males. Eight mice receiving 25-percent stilbestrol pellets developed mammary carcinomas at an average age of 10.8 months.*

PLATE 5



A, Gross mount of mammary gland of a normal strain C female 11 months of age. ×35; *B*, gross mount of mammary gland of an 11-month-old strain C female, an offspring of a strain C female that was foster nursed by a C3H female. ×35.

bestrol, revealed no significant differences in the morphologic appearance of the ovaries, adrenals, uteri, testes, and the accessory genital organs. The differences in these organs, described in comparisons of mice of high-mammary-cancer strains with animals of strains resistant to tumors of the breast (*9*, *14*), are therefore interpreted as being strain peculiarities that are not causally related to the disposition of the mice to mammary carcinoma. It has been pointed out (*16*) that reciprocal crossing or foster nursing offer much more equivalent bases for the correlation of morphologic differences to susceptibility of the animals to mammary cancer than do comparisons of genetically different strains.

Van Gulik and Korteweg (*17*) noted that foster nursing of strain dba mice (susceptible to tumors of the breast) by strain C57 Black mice (resistant to the development of this neoplasm) had a marked effect upon the architecture of the mammary gland in that it decreased the degree of alveolar arborization. Conversely, foster nursing of strain C57 Black mice by dba females increased the proliferation of the mammary alveoli. Preliminary studies at the National Cancer Institute corroborate this essential observation. Plate 5, *A*, shows the typical gross structure of the mammary gland of a normal strain C female 11 months of age. Plate 5, *B*, depicts the architecture of an 11-month-old strain C female, the offspring of a C mouse that was foster nursed by a strain C3H female. The greater proliferation of the alveoli and the nodules of hyperplastic tissue in the foster-nursed animal are obvious and striking.

SUMMARY

1. An adequate method of single administration of estrogens to mice for experiments in carcinogenesis was developed, using 4-to-6-mg. pellets containing 10 to 25 percent of stilbestrol in cholesterol.

2. Thirteen primary interstitial-cell tumors of the testis were induced in strain C mice following subcutaneous implantation of stilbestrol-cholesterol pellets. Three of the tumors metastasized to the region above the left kidney, and one also to the lung.

3. The appearance of lymphoid tumors was accelerated in strain C mice following the subcutaneous implantation of stilbestrol-cholesterol pellets.

4. Male and female mice of strain C were resistant to the induction of mammary carcinomas following treatment with stilbestrol. The incidence of mammary tumors rose to about 50 percent when the mice were nursed by C3H females and received stilbestrol pellets.

5. Foster nursing exerted no influence upon the induction of interstitial-cell hyperplasia and tumors of the testis, or upon the occurrence of brown degeneration of the adrenals, or upon the histologic appearance of the ovary in strain C mice.

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Sarcomas at Sites of Subcutaneously Implanted Bakelite Disks in Rats

By FLOYD C. TURNER, *senior surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

On November 6, 1936, a bakelite [1] disk, which had been coated with a very thin layer of 1, 2, 5, 6-dibenzanthracene by a vacuum distillation process, was implanted subcutaneously into the right side of an albino stock rat. An uncoated bakelite disk was implanted similarly into another albino rat, of undetermined origin, as a control. The test disk had been prepared by Dr. Egon Lorenz, of this Institute, for a study of the biological action of thin layers of carcinogenic hydrocarbons. Twenty days later the test disk was removed from the animal in order to observe the effect on the layer of hydrocarbon. No visible change was evident. The disk was not reimplanted. This rat died without tumor 11 months later, of cerebral abscess.

The control rat bearing the uncoated bakelite disk was killed 23 months after the disk had been implanted. A tumor surrounding the bakelite disk measured 21 x 33 x 49 mm. Dr. Hugh Grady, of this Institute, examined the tumor and reported that it was—

> an atypical fibrosarcoma composed of small bundles or nests of cells which varied from spindle to bizarre round or pear-shaped elements with large hyperchromatic nuclei. There was a heavy network of collagen fibers closely attached to many of the tumor cells, and small numbers of irregular, poorly formed blood vessels were seen. In phosphotungstic acid haematoxylin preparations fibroglia fibrils were identified in many tumor cells. Mitotic figures were numerous.

Transplants of this tumor into two Wistar strain rats did not grow.

Several investigators (*1–3*) have tested formaldehyde under varying conditions. None reported tumors following its use. No record was found to show that sodium sulfite, phenol, paraform, or orthocresol had been tested for tumor formation. Paracresol, which is chemically somewhat similar to orthocresol, was tested by Reimann (*4*), who made repeated applications to the skins of both rats and mice and observed no tumors after 6 weeks. Hexamethylenetetramine has been injected repeatedly into male mice by Dr. Shear and Dr. Stewart, of this Institute. [2] No tumors were observed in 5½ months. Bogen and Loomis (*5*) observed one benign tumor after having painted the skins of white mice for more than a year with caustic soda. Twort and Fulton (*6*) made repeated applications of lactic acid to the skins of mice and observed no tumors in 25 weeks. Petroff and Krotkina (*7*) used nine rats into which they made injections into two sites. Into one site was implanted embryonic tissues, and into the other, embryonic tissues plus lactic acid. Tumors occurred at the sites of the embryonic pulp, and in one rat a tumor arose at the site of the embryonic tissues plus lactic acid. Nakahara and Mori (*8*) fed glycerin to rats for more than 300 days. They observed neither tumors nor any other significant pathological condition. Hence,

[1] *Bakelite is formed by the chemical interaction between phenol and formaldehyde. In some products, a crude phenol, orthocresol, is used, and in others, paraform, a polymer of formaldehyde, is employed. As catalysts, the following substances have been used: caustic soda, sodium sulfite, hexamethylentetramine, lactic acid, and glycerin.*

[2] *Shear, M. J., and Stewart, H. L.: Unpublished data.*

no very conclusive evidence of carcinogenic activity was obtained by any of the ingredients of bakelite, by the methods reported.

EXPERIMENTAL PROCEDURE

Experiment 1

On October 4, 1938, squares of bakelite 10 x 10 x 1½ mm. were implanted subcutaneously into the right sides of 10 male strain dba mice 2½ months old. The last mouse died at the age of 18 months. No tumors were observed in any of the mice. There was no apparent change on the surface of the bakelite squares.

Experiment 2

On December 16, 1938, a bakelite disk was implanted subcutaneously into the right side of each of six male and six female Wistar strain rats 6 weeks old. The bakelite disks were 1½ mm. thick and 18 mm. in diameter. The surface of each disk had been removed with a new steel file after which the disks were scrubbed with a brush, soap, and hot water, rinsed in tap water, boiled in distilled water, and implanted.

Several of the rats died early of pneumonia. The first palpable mass at the site of a bakelite disk was observed 18 months after the disks had been implanted. Eight rats, five males and three females, were alive on that date. Of these eight rats, three developed tumors at the sites of the bakelite disks.

Figure 1.—Gross appearance of tumor which developed around a bakelite disk. The other tumors had a similar appearance.

Figure 2.—Fibrosarcoma showing bundles of spindle-shaped cells. Phosphotungstic acid haematoxylin stain. ×350.

The pathological diagnoses of these were also made by Dr. Grady. He found that—

all three tumors were fibrosarcomas and were composed of interweaving bands of long, spindle-shaped cells with tapering processes and darkly staining nuclei. Moderate amounts of collagen were present in two instances, while in the third tumor large collagenous bundles were observed. Fibroglia fibrils were easily identified in all three tumors and mitotic figures were present.

No attempt was made to transplant the tumors (figs. 1 and 2). In three tumors a brownish-orange-colored fluid was in contact with the bakelite disk. In one instance, the disk was inclosed in a cyst containing this fluid. Histopathological examination of this fluid by Dr. Grady

revealed large numbers of mononuclear cells. A spectrographic examination of the fluid by Dr. Herbert Kahler, of this Institute, revealed only components of the hemoglobin spectrum.

DISCUSSION

Disks were implanted into 13 rats. Nine of the rats lived to be 20 months of age, or older; tumors developed in 4, 3 males and 1 female. Bakelite squares were implanted into 10 mice. Six of the mice lived to be 18 months old and had had the bakelite constantly in contact with their subcutaneous tissues for 15 months. This period of time was probably as great a fractional part of the life span as that experienced by the rats, yet tumors developed in the rats but not in the mice. Although the number of test animals was small, the findings suggest that the rats were more susceptible to tumor formation by this agent. What factors underlie this difference are not known; both were fed the same diet, dog chow, except that the rats were occasionally given cod-liver oil because they showed evidence of vitamin A deficiency. This dietary deficiency was not studied in relation to tumor formation.

A tumor, a mammary adenofibroma, developed at the base of the tail of the last surviving rat (female) which lived to be 26 months old. It probably was a spontaneous tumor which arose uninfluenced by the presence of the implanted disk. No tumor was found at the site of the bakelite disk.

It required many months for the tumors to develop around the disks, but they grew rapidly after their initial appearance. There appeared to be very little, if any, erosion of the bakelite disks.

The mode of action of the biological processes involved in the formation of tumors by bakelite is as ambiguous as the *modus operandi* of other carcinogenic agents. Although tumors occurred in the rats and not in the mice, chronic irritation cannot be entirely excluded as an adjuvant factor, for the subcutaneous tissues of the rat may be more favorable to tumor formation than the subcutaneous tissues of the strain of mice employed.

The fact that all of the mice and most of the rats bore the disks continuously in contact with their subcutaneous tissues for most of their entire lifetimes without developing tumors suggests a very low hazard of carcinogenicity.

SUMMARY

Bakelite disks were implanted subcutaneously into 13 rats. Of 9 rats which survived longer than 20 months, 4 developed sarcomas at the site of the disk.

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The Problem of Gastric Cancer[1][2][3]

By WILLIAM A. COOPER, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The prodigious effort expended by the medical profession in the study of malignant diseases probably has had less effect on mortality from gastric cancer than on that from any other of the common types of carcinoma. While gratifying progress has been made in the treatment of cancer of the skin, nasopharynx, larynx, cervix uteri, and the breast, cancer of the gastrointestinal tract, and most particularly of the stomach, remains a highly lethal disease. A favorable outcome in gastric cancer is so unusual that most practicing physicians have never witnessed a cure. Indeed, only those privileged to observe, over a long period of time, large numbers of patients with cancer of the stomach are in a position to know of the occasional cures. In a disease with such a dismal outlook it seems prudent to review its salient features to determine (1) if this outlook is justified and (2) what is to be done to correct the situation.

To illustrate the problems, the experience of the New York Hospital in cancer of the stomach since September 1932 is analyzed. It is recognized that the number of patients who have passed the 5-year period is small, a factor which may somewhat distort the end results. It is hoped that there may be occasion to present this same material from time to time, modified by further experience with the same cases and augmented by new cases studied in the interim.

CLINICAL MATERIAL

During the 7⅓-year period from September 1932 to 1940, 264 patients with proved cancer of the stomach were admitted to the medical and surgical pavilions of the New York Hospital. It is felt important to include both the medical and the surgical cases to portray a well-rounded picture of the disease as it is encountered in a large general hospital. In 88 of the cases (33⅓ percent) the disease was sufficiently advanced to be considered inoperable clinically. In 91 cases (34.5 percent) an exploratory laparotomy was done, with 7 deaths occurring in the hospital after exploration, an operative mortality of 7.7 percent. In 21 cases (7.9 percent) a palliative gastroenterostomy was done, with 5 operative deaths, a mortality of 23.8 percent. In 7 cases (2.7 percent) miscellaneous procedures, such as colostomy, gastrostomy, or total gastrectomy were done, with 6 operative deaths (85.5 percent). In 16 cases (6.1 percent) a palliative gastric resection was done in the presence of metastasis which could not be removed, with 2 operative deaths (12.5 percent). Only 41 out of the total of 264 cases (15.5 percent) were sufficiently early to be considered curable by surgical methods. In these a partial gastrectomy was done, with 4 operative deaths, an operative mortality of 9.8 percent. The combined mortality for both the palliative and curative resections was 10.5 percent. These data are shown graphically in the accompanying chart.

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[1] *From the Department of Surgery of the New York Hospital and Cornell University Medical College, carried out under a grant from the National Cancer Institute of the U. S. Public Health Service, Washington, D. C.*

[2] *This article was written for the purpose of familiarizing practicing physicians with the subject of cancer.*

[3] *Reprinted by permission of the Journal of the American Medical Association, from volume 116, No. 9, pp. 2125–2129, May 10, 1941.*

ONLY 41 CASES (15.5 %) FAVORABLE FOR CURATIVE OPERATION
CURATIVE GASTRIC RESECTION
PALLIATIVE GASTRIC RESECTION
PALLIATIVE GASTROENTEROSTOMY
OTHER PALLIATIVE OPERATIONS
INOPERABLE BY EXPLORATORY LAPAROTOMY
264 CASES OF GASTRIC CANCER
CLINICALLY INOPERABLE
223 CASES TOO ADVANCED FOR HOPE OF CURE

RESULTS

All of the 223 patients (84.5 percent) with cancer too advanced for hope of surgical cure were doomed to die. The average duration of life after admission to the hospital in this group is given in table 1.

TABLE 1.—*Duration of life, in months*

Not operated upon	4.8
Exploratory laparotomy	6.5
Palliative gastroenterostomy	11.0
Palliative gastric resection	9.3

The variation in the duration of life in these groups is largely explained by the extent of the gastric lesions when the patients were admitted to the hospital. Obviously, those patients with cancer sufficiently advanced to be clinically inoperable should live a shorter time than those found inoperable only by exploration. Though the duration of life after palliative resection or palliative gastroenterostomy is not strikingly prolonged, there is no question concerning the value of these procedures. Most of the palliative gastroenterostomies were performed for pyloric obstruction, and there is no doubt that some months were added to the duration of life in this group. Vastly more important than the few months of life, however, is the fact that in general this group was clinically improved by gastroenterostomy. In most instances there was a cessation of vomiting, an increase in appetite, and some gain in weight. Two patients of this group enjoyed 2 years of practically normal life.

Clinical improvement in the group of palliative gastric resections was even more striking, though the average duration of life was not quite as great as that in the palliative gastroenterostomies. Many of these patients were able to lead essentially normal lives until a few months before death. The usual prolonged period of disability was greatly shortened, and the final period of decline was

relatively rapid. So general was the improvement in the palliative resection group that this procedure usually is felt to be indicated when it is mechanically possible to remove all of the gastric tumor.

Of the 41 patients on whom curative gastric resections were performed, 15 are now dead, the average duration of life after operation being 16 months. Only 9 patients had curative resections done 5 or more years ago. Of these, 4 are living and free from disease, a 5-year survival rate of 44.4 percent. Of the 17 patients on whom curative resections were done 3 or more years ago, 10 are living and free from disease, a 3-year survival rate of 58.8 percent.

These figures are sufficiently striking to warrant scrutiny. They are open to question in two ways: (1) In separating the curative and palliative resections there has been some selection of cases. If the end results are calculated for all resections, the 3-year survival rate is 52.6 percent and the 5-year survival rate 40 percent—figures which are still unusual for cancer of the stomach. It is scarcely justifiable, however, to compromise gastric resection as a method of treatment by including in the curable group those cases of metastasis which obviously could not be removed at operation. (2) A second, more valid criticism is that the series is small. The 3-year survival rate, however, indicates that the 5-year survival rate in this series will probably remain elevated for at least 2 years to come.[4]

[4] *This is not the only series in which the end results have been unusual. Pack and Livingston (End Results in the Treatment of Gastric Cancer, New York, Paul B. Hoeber, Inc., 1939, p. 127) collected about four thousand gastric resections done for cancer in which the 3-year survivals averaged 34.6 percent, the 5-year survivals 26.8 percent and the 10-year cures, when reported, 19.66 percent, i. e., roughly one-third living at 3 years, one-fourth living at 5 years and one-fifth living at 10 years. These averages include 5-year survivals of 39.5 percent reported by Gatewood, 33.3 percent reported by Harms, and 28 percent reported by Balfour.*

The experience of the New York Hospital, therefore, indicates that operable cancer of the stomach is cured in 44.4 percent of cases for a period of 5 years by gastric resection, a result comparable to that in cancer of the breast, a disease generally regarded as curable. Surely, then, the despondent outlook on gastric cancer is not justified. But this optimistic side of the picture is seen only by the surgeon who follows operable cases. The physician in general practice is likely to be more impressed by a broader picture, that these four cures represent only 5.4 percent of the 72 patients admitted to the hospital 5 or more years ago, a picture that emphasizes the rarity of the cures of gastric cancer.

TREATMENT

No treatment other than the surgical treatment of gastric cancer is worthy of mention. In view of the basic pathologic condition (neoplastic infiltration of a hollow viscus) it seems likely that roentgen therapy will never furnish a rational attack on the disease. Still the record of surgical intervention as a method of treatment is so poor that cures for the group as a whole are distinctly rare. It is not to be assumed that surgery has made no progress in its attack on the disease. During the 60 years since Billroth performed the first successful partial gastrectomy, the mortality of the operation has been reduced from a formidable figure to about 10 percent or less in the hands of competent surgeons. It is clear that this figure will be further reduced by continued concentrated effort. But this progress has been largely confined to making gastric resection a safer operation. The physician cannot rely on the surgeon to improve the outlook on gastric cancer by further refinements in surgical technic. Not until more resectable cases are seen by the sur-

geon will cures of this dread disease become common. It is clear, therefore, that the essence of the problem of gastric cancer is that of early diagnosis.

One should therefore examine the available methods that make diagnosis possible to determine wherein these methods contribute to the timely discovery of cancer of the stomach.

1. *The History and Physical Examination.*—The histories and physical findings presented by this group of patients were in 84.5 percent of the cases those of inoperable gastric cancer. The typical picture with upper abdominal pain or discomfort, progressive anorexia and loss of weight, vomiting, anemia, and weakness was sufficiently striking in most of the cases to be recognized by the uninitiated. If the patient seeks medical advice only after this stage has been reached, in the majority of cases he is merely delegating to his physician the unpleasant task of terminal care, a task for which the philosopher or the minister of God may be as well suited as the student of medicine. The tragic drama of inoperable gastric cancer confirms in the minds of the patient, his friends, and his relatives a horror of cancer in general and further frustrates his physician. It is relatively unimportant to the patient that the clinical picture of inoperable gastric cancer be recognized. In fact, most of the clinical experience with gastric cancer is futile unless it teaches doctors how to diagnose the disease earlier.

There seems little hope that the casual, superficial history will discover gastric cancer in the operable stages. Mullen [5] has aptly stated that "physicians might be divided into two classes: (1) Those who are eternally suspicious of gastric cancer and never find it; (2) those who are never suspicious of it and eternally miss it. The first are apt to complain of the futility of their efforts, and the second to comment on the hopelessness of the disease." Certainly care, healthy optimism, and suspicion are as essential to the gastric diagnostician as are hands to the surgeon.

The early symptoms of gastric cancer can be summarized in the two indefinite terms frequently used by the layman—"dyspepsia" and "indigestion." Reduced to medical terminology these symptoms are as follows:

(*a*) Loss of appetite is one of the earliest and commonest symptoms. At first the patient notes that heavy foods he has previously eaten and enjoyed cause some upper abdominal discomfort, fulness or gas. He soon loses desire for these foods and eliminates them from his diet. In time he finds that other foods disagree with him, and he suffers from true anorexia. This frequently progresses to an abhorrence of all food—at which time the growth has usually gone too far for surgical removal.

(*b*) Fulness after meals often precedes by weeks or months the development of upper abdominal discomfort or pain. The appetite is satisfied more easily than usual, and the patient does not enjoy his favorite dish, which is often an indigestible concoction.

(*c*) The fulness disappears when less is eaten and the old favorites are eliminated from the diet but in time progresses into upper abdominal discomfort after meals, which in turn may develop into pain.

(*d*) Epigastric pain is not always present and in itself is more typical of ulcer than of cancer; this is particularly true if it is intermittent and responds to food and alkalis. In carcinomatous ulcers, however, pain may be present from the outset and may exactly simulate ulcer pain. Mild pain without periodicity may occur both early and late in the disease.

[5] *Mullen, T. F.: Some Factors Influencing the Curability of Cancer of the Stomach, Surg., Gynec. & Obst. 72: 298 (Feb., no. 2A) 1941.*

(*e*) Gas and belching are frequently associated with the symptoms listed or they may occur alone as the first symptoms of gastric cancer. If the gas tastes foul to the patient an advanced necrotic tumor is usually present.

(*f*) Nausea, particularly after eating, is often present early, though advanced cases have been observed in which this sensation has never been experienced. The nausea may occur alone or may be associated with vomiting.

(*g*) Vomiting, if persistent and severe, is more often a late than an early symptom. Occasional unexplained attacks of nausea and vomiting followed by complete relief are frequently the first symptoms of gastric cancer. The patient invariably refers such an attack to a dietary indiscretion. If the emesis contains changed blood, the disease is likely to be advanced.

The symptoms enumerated attract the attention of both the patient and the physician to a gastric disorder. However, all these symptoms may be entirely absent and the physician may have some difficulty in knowing to which system he should attribute the nonspecific symptoms that follow.

(*h*) Weakness, lack of vigor and strength, or of the usual sense of well-being, may be the first manifestations of gastric cancer. Though usually related to either loss of weight or anemia, they may occur alone as the earliest symptoms.

(*i*) Anemia, often associated with weakness and pallor, is sometimes the first sign of the disease but is more characteristic of advanced gastric cancer. The typical picture of pernicious anemia is unusual but is seen often enough to warrant a complete investigation of the stomach before liver therapy is started. In view of the disproportionately high incidence of gastric cancer in patients with proved pernicious anemia, and of the possible relation of atrophic gastritis to cancer of the stomach, it is imperative that patients known to have either pernicious anemia or atrophy of the gastric mucosa be thoroughly reinvestigated every six months as long as they live, regardless of their response to liver or other therapy.

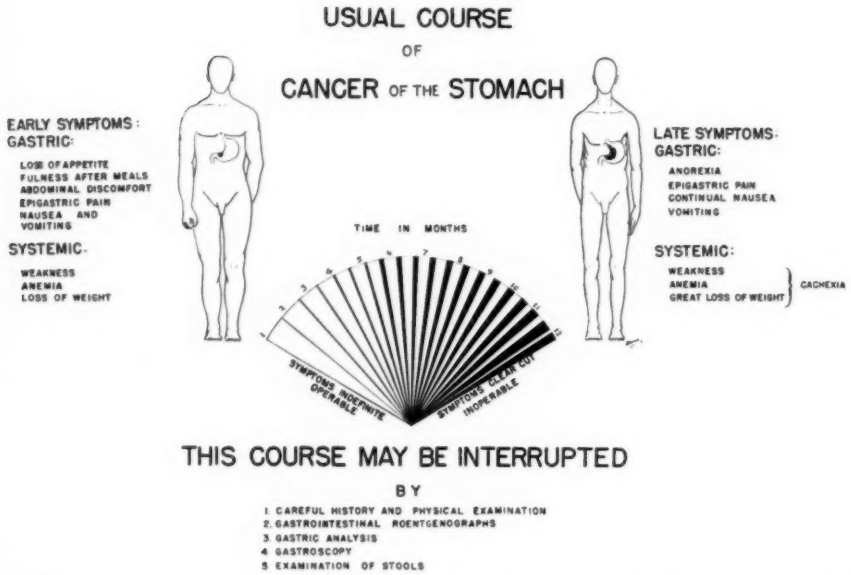
(*j*) Loss of weight, while characteristic of advanced disease, may occur alone or associated with the other nonspecific symptoms, as the first sign of the disorder. This is particularly true of patients lacking sensitivity to symptoms in general.

Abnormal physical findings are usually entirely absent in early gastric cancer. Slight epigastric tenderness or resistance may be elicited on examination; this is especially common in cancer that simulates ulcer.

A fixed epigastric mass is usually advanced and inoperable, but a movable mass may be a bulky adenocarcinoma which can be resected with a fairly favorable prognosis in spite of the terminal signs of disease. Because of this type of cancer, a movable lesion should be explored before it is declared to be inoperable regardless of its size.

Though a careful physical examination is essential, it is the history which is of greater importance in the discovery of early cancer. Only by carefully planned questioning regarding former dietary habits to contrast with the present reactions to food can the physician form a picture on which to base his diagnosis.

It is often stated that cancer does not develop in a normal stomach. This is most apparent in the 25 percent of patients who for many years have suffered from mild gastric symptoms some or all of which simulate the early symptoms of gastric cancer. Clinical diagnosis is particularly difficult in this group, for the changes in the reactions of these patients to food may be extremely subtle.



The average duration of symptoms before the patients entered the hospital in this series was 12½ months. Strangely, the duration of symptoms of the 41 patients with resectable cancer was 16 months, 3½ months above the average. Difficulty was encountered in attempting to place the responsibility for the delay of more than 1 year between the onset of symptoms and the establishment of a positive diagnosis, for the hospital records frequently omit detailed accounts of the medical experiences of patients before entering the hospital. In 88 case histories these data were recorded. As one might anticipate, analysis shows that both the patient and the private physician contributed to the delay. The average delay on the part of the patient in seeking medical aid was 8 months, while the average delay on the part of the private practitioner in establishing the diagnosis was 4½ months.

A more accurate and dramatic study of the responsibility for delay in the diagnosis can be made by examining the records of the out-patient department. In spite of readily available diagnostic aids, in a number of instances there was delay of 6 months or more in establishing the diagnosis. Responsibility for this delay is squarely on the shoulders of the out-patient department and emphasizes the inadequacies of our present attack on the disease.

It is apparent, therefore, that the history and physical examination, though primarily essential, fail to establish the diagnosis in many instances and, as applied by both the general practitioners and the physicians of the clinic, allow for a delay of 1 to 6 months in bringing the patient to surgical treatment.

Unfortunately the vagueness of the early complaint and obscurity of the physical findings more often than not render early clinical diagnosis difficult, if not impossible. The profession, therefore, has come to rely on various special examinations to compensate for the inaccuracies of the usual clinical methods. Procrastination on the part of the physician in delaying further

TABLE 2.—*Reports of Roentgen Examinations*

	Examination					Final Diagnosis	
	1st	2d	3d	4th	5th		
Positive reading of cancer of the stomach	70.7%	83.0%	84.7%	86.0%	86.0%	86.0%	
Questionable, suspicious of cancer of repeat examination requested	11.8%					3.1%	33 cases*
Diagnosed as other pathologic condition, i. e. ulcer, gastritis, spasm, or benign lesion.	13.1%					8.7%	
Normal stomach	4.4%					2.2%	
	Total cases examined 229						

*In the group of 33 cases in which repeated roentgen examinations failed to clarify the final diagnosis, 29 patients had gastric acid studies, the results of which are shown in table 3.

investigation until the symptoms become well established accounts for a good portion of the inoperable gastric cancer seen at the New York Hospital.

2. *The Roentgenographic Examination.*—Certainly the most useful of the diagnostic aids in cancer of the stomach is the roentgenographic examination. Without fluoroscopic and roentgen studies it is impossible to advise patients properly regarding their gastric complaints. But just how accurate is this examination in the diagnosis of gastric cancer, and is it to be relied on to diagnose cancer of the stomach early? The existent impression is that the roentgenologist is extremely accurate in both the discovery and differential diagnosis of gastric lesions. Comfort and Butsch [6] found the roentgen diagnosis to be correct in 78 percent of gastric lesions of less than 4.4 cm. in diameter, while 12 percent of the diagnoses were equivocal and 10 percent were wrong. In diagnosing benign gastric ulcers they were 93 percent correct; in diagnosing malignant gastric ulcers they were 70 percent correct. The general impression is that the roentgenologist is about 90 percent accurate in the diagnosis of cancer of the stomach.

In critically analyzing the roentgenographic reports on my group of cases it is found that the accuracy approaches the commonly accepted 90 percent when repeated examinations are possible. The reports of these examinations which were returned to the clinicians are shown in table 2.

It is apparent from table 2 that the roentgenologist is certain of only 70.7 percent of the cases on the first examination but that with repeated examinations the accuracy goes up to 86 percent. If one adds the 3.1 percent still suggestive of cancer with repeated examinations, the accuracy is 89.1 percent. In the last analysis only 2.2 percent of this entire group were said to have some type of pathologic lesion.

Since it is most important for the clinician to be acutely aware of the diagnostic limitations of gastric roentgenograms it is essential that he bear in mind the 30 percent suggestive, benign or negative diagnoses returned after the first examination rather than the remarkable 97.8 percent final accuracy in detecting disease. It is also important for the clinician to remember that this accuracy in roentgenologic diagnosis was obtained in a group of cases in which 84.5 percent were inoperable. It is likely that the roentgen examination would be much less accurate in a group of cases with higher operability. In this series the roentgenologist diagnosed cancer in 80 percent of the 41 resectable cases. Intelligent correlation of the clinical history with the roentgenologic report

[6] *Comfort, M. W., and Butsch, W. L.: Differential Diagnosis of Benign and Malignant Small Lesions of Stomach, Am. J. Surg. 35: 515 (March) 1937.*

is of paramount importance in establishing a diagnosis of gastric cancer. A carefully taken history compatible with cancer of the stomach should probably never be overruled by a single negative roentgen report. Further study is usually indicated.

3. *The Gastric Analysis.*—Considerable confusion exists regarding the value of the gastric acidity as a diagnostic aid in cancer of the stomach. There is little question that much of this confusion has arisen through the use of a variety of technics and inaccurate performance of the test. In measuring the gastric acidity it is important to remember that one wishes to know how much acid the stomach is capable of producing. To obtain a maximum response it is necessary to use histamine as a secretagogue; to measure this response it is necessary continuously to aspirate the gastric juice (and gastric juice only). Errors in the test creep in through the aspiration of bile, the swallowing of saliva and failure to aspirate continuously. Using the technic described by Bloomfield and Polland,[7] Holman [8] has carefully studied the gastric acidity in the New York Hospital series. He found the acid below normal or absent in 85 percent of the cases of gastric cancer and to date has found no example of hypoacidity in gastric ulcer. In this clinic, therefore, the gastric analysis has been found a useful aid in the diagnosis of cancer of the stomach. Its chief value lies in differentiating benign and malignant lesion. Though fallible in 15 percent of the cases, it adds confirmatory evidence in 85 percent of cases of gastric cancer. In the absence of a gastric lesion, the diagnostic value of the gastric analysis has not yet been determined. An effort is being made to determine its value by doing the test on all patients with gastric symptoms. Diagnostic aids are so urgently needed in cancer of the stomach that a complete study should be employed in all patients suspected of having the disease.

[7] *Bloomfild, A. L., and Polland, W. S.: The Diagnostic Value of Studies of Gastric Secretion, J. A. M. A. 92: 1508 (May 4) 1929.*

[8] *Holman, Cranston: The Diagnosis of Gastric Carcinoma and Peptic Ulcer. J. A. M. A. 108: 1383 (April 24) 1937.*

TABLE 3.—*Results of Studies on Gastric Acidity in Cases Not Diagnosed by Roentgen Examination*

Free acid 0°	13 cases	65.5%
Free acid below 50°	6 cases	
Free acid above 50°	10 cases	34.5%

The reduced or absent free hydrochloric acid in 19 of the 29 cases supported the diagnosis of gastric cancer, leaving but 10 cases (3.7 percent) out of the entire series in which neither roentgen examination nor determination of the gastric acidity tended to confirm the clinical picture which made these studies necessary. On scrutiny it is found that 9 of these 10 cases were diagnosed by the roentgenologist as gastric ulcer. This simply confirms the experience of most observers that it is difficult to differentiate with certainty the benign from the malignant ulcer.

The questionable lesions that the gastric analysis fails to place in the malignant group present a real diagnostic problem. Yet this problem is simplified if one considers surgery as indicated in any gastric ulcer that fails to heal under conservative treatment. If the ulcer is chronic and has recurred repeatedly under adequate treatment over a period of years, gastric resection usually is indicated whether the lesion is benign or malignant. The recent gastric ulcer with a short history may be given a 2-week period of conservative care in the hospital. If the roentgenograms fail to show a marked diminution in size or if the clinical symptoms are not alleviated, resection again should be considered. It is not safe, however, to regard reduction

in size or the disappearance of symptoms as proof of the benignity of a gastric lesion, for malignant ulcers have been known to disappear on the roentgenogram and the patients commonly improve clinically on bland diets. Such cases deserve continued observation until the physician is satisfied that the healing is complete and permanent. Among all others, ulcers of the prepyloric area should be regarded with the greatest suspicion.

4. *Gastroscopy.*—The most recent addition to the diagnostic armamentarium of gastric disease is the flexible gastroscope designed by Wolfe and Schindler in 1932. While statistical data regarding its value in the diagnosis of gastric cancer are lacking, there can be no question that certain tumors missed on roentgen examination are visible through the gastroscope. If this occurs with sufficient frequency, the operability of cancer of the stomach will certainly be increased. An increase will be observed, however, only if the examination is applied to a large group of patients suspected of having gastric cancer. In the diagnostic problem of gastric cancer the real need for gastroscopic examination is in those cases in which the roentgenogram fails to establish the diagnosis. Since it is apparent that roentgen examination has limitations which increase the earlier the disease is seen, gastroscopic examination will in all probability become increasingly necessary as a diagnostic aid.

5. *The Stool Examination.*—A less specific but nonetheless useful diagnostic aid is the examination of the stools for occult blood. Any oozing or ulcerating lesion of the gastrointestinal tract will, of course, give a positive reaction. Occult blood in the stools necessitates further study to determine its origin and cause and therefore can be added to the group of symptoms considered under the history and physical examination which demand a clear explanation. Though occult blood is usually present in the stools of patients with gastric cancer, unfortunately its absence does not exclude the disease, for scirrhous carcinoma may not ooze or ulcerate.

6. *The Importance of Complete and Repeated Examinations.*—Most of the delay in diagnosis in the New York Hospital out-patient department was due to a false security derived from negative reports on one or more of the diagnostic aids. In several instances, after a negative roentgenologic examination the patient was reassured and lost contact with the clinic until some months later when the symptoms were well advanced. It is of paramount importance to carry out a complete gastric investigation on every patient over 35 years of age who has symptoms compatible with early gastric cancer. Nor is it sufficient for one to complete the investigation and, finding it negative, discharge the patient as well. The proper management of such a case demands continuous observation until all the early symptoms have disappeared. If they do not disappear, a complete reinvestigation should be done at frequent intervals until the diagnosis whatever it may be, is established. In this method of management lies a fertile field of endeavor against cancer of the stomach. Not until these concepts are accepted and practiced by every physician who sees patients with gastric complaints will the profession be performing its function against this dread disease.

SUMMARY

The despondent outlook generally held by physicians toward cancer of the stomach is justified only by the high proportion of failures (95 percent) experienced in the past. Analysis clearly shows that the failure is largely due to late diagnosis. It also shows that the responsibility for the delay in diagnosis is divided among the patient (8

months), the patient's physician (4½ months) and the general hospital (1 to 6 months). But the profession in its fatalistic outlook based on past experience should not accept these figures as irreducible. With highly dependable diagnostic aids available there is every reason to believe that their vigorous and repeated application would reduce the professional delay to the vanishing point. With proper educational steps the patient's delay could be reduced materially. The common expression of the profession "They all die anyway" reflects an unhealthy attitude of despair which must give way to an enthusiastic and vigorous attack on the disease before real progress is possible. It is firmly believed that a complete reversal of attitude toward cancer of the stomach on the part of the medical profession is long overdue.

The Effect of Dietary Cystine on the Reaction of Dilute Brown Mice to Methylcholanthrene[1] (Preliminary Report)

By JULIUS WHITE, *research fellow*, and G. BURROUGHS MIDER, *research fellow*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

It has been shown that young growing rats failed to gain weight when they ingested a low cystine diet containing methylcholanthrene (*1*, *2*). The addition of sulfur-containing amino acids to the diet caused a prompt stimulation of growth. These observations suggested that the addition of the sulfur-containing amino acids resulted in detoxication of the methylcholanthrene.

Experiments were designed to study the effect of availability of sulfur-containing amino acids on the induction of leukemia by methylcholanthrene in mice of the dilute brown (dba) strain (*3*, *4*, *5*). A synthetic diet was devised which contained adequate amounts of all known substances necessary to growth excepting the sulfur-containing amino acids, cystine and methionine.

It had the following composition:

	Percent
Casein (vitamin-free)	3. 9
Starch	55. 1
Crisco	22. 0
Cod-liver oil	3. 0
Salts (modified Osborne-Mendel)	6. 0
Ryzamin-B (Burroughs Wellcome)	5. 0
Liver extract (Lilly 343)	5. 0
	100. 0

The concentration of sulfur-containing amino acids in the diet was sufficient for maintenance of life but did not permit weight increment.[2] Mice fed this diet had rough coats, failed to gain weight, and did not breed; but no structural changes were found in their organs or tissues after they had eaten it for more than 6 months. When 0.5 percent l-cystine was added to the food, the mice gained weight, and the females bore litters of average size. Little difference could be noted between mice ingesting the cystine-supplemented diet or Purina dog chow.

Young litter mates of dilute brown mice of subline 212 were divided into 3 groups One group was fed Purina dog chow; another, the low cystine diet; and the third group, the high cystine diet. All mice were painted with a 0.2-percent solution of methylcholanthrene in ethyl ether. The solution was painted on the skin on alternate days until the mouse died. The painting site was changed with each application as in previous experiments (*3*, *4*). Other dilute brown mice ingesting the high or low cystine diet painted in the same manner with ethyl ether for 100 days served as controls.

RESULTS

The results are summarized in table 1. No macroscopic lesions were found among the control mice that were painted with ether only.

The leukemias were similar in gross and histologic appearance to those previously described in dilute brown mice painted with solutions of methylcholanthrene (*4*).

The mice that ingested the low cystine diet and were painted with the 0.2-percent

[1] *Presented at the sixth annual meeting, American Association for Cancer Research, Chicago, Ill., April 15, 1941.*

[2] *Unpublished data.*

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TABLE 1.—*Effect of diets on response of dilute brown mice to methylcholanthrene*

Diet	Total number of mice	Mice with no neoplasm	Leukemia			Sclerosis of aorta		
			Number	Percent	Mean latent period	Number	Percent	Mean latent period
					Days			*Days*
Dog chow	19	1	17	89.4	81	0		
High cystine	39	4	35	92.3	81	1	2.6	92
Low cystine	35	29	6	17.1	91	33	94.2	80

solution of methylcholanthrene in ethyl ether developed sclerosis of the larger blood vessels. It was most marked in the arch and thoracic portions of the aorta where thick, yellow-white, opaque, calcified rings encircled the vessel (fig. 1). Some of the lesions were confluent (fig. 2). Sclerotic aortas did not collapse when they were transected. The carotid, renal, and cerebral arteries and the larger arteries in the splanchnic area sometimes appeared as milky-white, opaque threads but they were involved less frequently and less extensively than the aorta. No gross changes were seen in the heart.

Large deposits of amorphous calcium replaced the normal architecture of the media and caused local thickening of the walls of the sclerosed arteries (fig. 3). The calcific plaques sometimes extended throughout the circumference of the media,

FIGURE 1.—Thoracic aorta. Deposits of calcium salts form white bands in the wall of the vessel. ×3.

FIGURE 2.—Thoracic aorta. The calcium deposits form confluent patches. ×3.

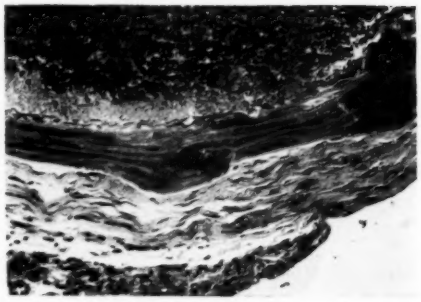

FIGURE 3.—Abdominal aorta. Amorphous deposits of calcium salts destroy the architecture of the media. The adventitia is thickened. Haematoxylin and eosin. ×150.

but longitudinal sections of the vessels showed a segmental distribution. The media between plaques looked relatively normal although small foci of degenerated muscle cells were seen occasionally. No evidence of cholesterol deposits could be found. In sections stained by Verhoeff's method for elastic tissue the elastic fibers appeared normal in the areas between the calcific deposits but disappeared in the plaques (fig. 4). In some areas of the aorta the continuity of the elastic fibers was interrupted by masses of collagenous tissue continuous with the adventitia. These extended to the endothelial layer and appeared as dense scars.

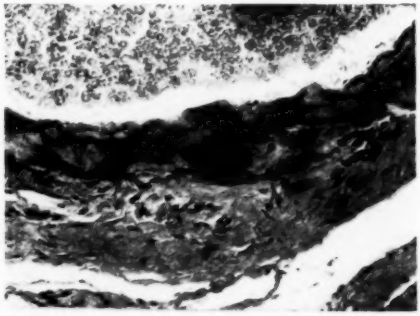

FIGURE 4.—Thoracic aorta. The elastic laminae are lost in the calicific plaque. The adventitia is thickened. Verhoeff's elastic tissue stain and van Gieson's stain. ×250.

The adventitia of the sclerosed vessels was markedly thickened. It frequently contained small collections of lymphocytes and other mononuclear cells.

No histologic changes were noted in small arteries, veins, or lymphatics. Small foci of myocardial degeneration were seen in some of the mice, but similar areas have been found frequently among mice painted with methylcholanthrene solutions regardless of diet.

The spleens from the arteriosclerotic mice were atrophic, as indicated by marked reduction in the number and size of lymphoid follicles. Cells in the splenic pulp contained yellow-brown, granular pigment. Some of the males showed atrophy of the germinal tubules of the testes which was always focal in distribution. Other organs and tissues contained no noteworthy changes.

The reduction in the incidence of leukemia among the mice that ate the low cystine diet may be more apparent than real. Mice with arteriosclerosis died at about the same time that other mice developed palpable enlargement of the lymph nodes, a valuable sign in the early diagnosis of most methylcholanthrene-induced leukemias. The leukemic lesions among the arteriosclerotic mice were found in those animals that survived the longest.

The absence of arteriosclerosis in control mice that ingested the low cystine diet for more than 6 months or in those that were painted with ethyl ether suggests that methylcholanthrene was responsible for the arterial changes observed. The reaction also appears to be contingent on the concentration of sulfur-containing amino acids in the diet since arteriosclerosis was found only among those mice that ingested the low cystine diet. It is conceivable that other dietary deficiencies might produce similar lesions. However, if one may assume that cystine is required for detoxication of methylcholanthrene, then these experiments suggest that the vascular damage is a manifestation of the toxicity of methylcholanthrene probably not related to its carcinogenic properties.

SUMMARY

The incidence of leukemia induced by methylcholanthrene in mice of the dilute brown strain was reduced when the concentration of cystine in the diet was decreased so that weight increment was prevented. Sclerotic lesions of the aorta and other large arteries occurred frequently among the mice that ingested the low cystine diet.

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